AD_____

Award Number: DAMD17-00-1-0527

TITLE: Green Tea in Prevention and Therapy of Prostate Cancer

PRINCIPAL INVESTIGATOR: Hasan Mukhtar, Ph.D.

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, Ohio 44106-7015

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020124 261

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Green Tea in Prevention and Therapy of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0527

6. AUTHOR(S)
Hasan Mukhtar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Case Western Reserve University
Cleveland, Ohio ~~44106-7015~~ 44106-7015
E-Mail: hxm4@po.cwru.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Green tea consumption has been associated with a decrease in the risk of some cancer types in humans. Epidemiological studies, though inconclusive, suggest that drinking green tea may lower the risk of prostate cancer (CaP) in humans. Here we report that polyphenolic mixture obtained from green tea [0.1% GTP (w/v) in drinking water] at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibits prostate cancer development and blocks metastases in **tr**ansgenic **a**denocarcinoma of the **m**ouse **p**rostate (TRAMP) mice. The cumulative incidence of palpable tumors at 32 weeks of age in water-fed TRAMP was 100%. However, GTP provided in drinking water to TRAMP mice from 8 to 32 weeks of age resulted in (a) significant delay in primary tumor incidence, (b) significant decrease in prostate and genitourinary weight, (c) significant inhibition in serum IGF-I and restoration of IGFBP-3 levels, (d) marked reduction in the protein expression of PCNA, and (e) increased apoptosis in the prostate compared to water-fed TRAMP mice. The striking observation of this study was that GTP infusion resulted in almost complete inhibition of distant site metastases. Furthermore, GTP consumption to TRAMP mice resulted in increased tumor free survival and prolonged life span of these mice.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Green tea, EGCG, prostate cancer, apoptosis, TRAMP	46
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents..... 3

Introduction..... 4

Body..... 4-8

Key Research Accomplishments..... 8

Reportable Outcomes..... 8-9

Conclusions..... 9-10

References..... 10-12

Appendices..... 13-46

INTRODUCTION:

Green tea, a popular beverage has received much attention in the recent past in reducing the incidence of certain human cancer. Extensive laboratory studies in cell culture systems and in animal models have demonstrated that green tea and its major polyphenolic constituent (-) epigallocatechin-3-gallate (EGCG) afford protection against many cancer types in experimental models. Epidemiological observations, though inconclusive, are suggesting that green tea consumption is associated with reduced risk of some human cancers. Based on the epidemiological evidence where Japanese and Chinese population consuming green tea on a regular basis have the lowest incidence of prostate cancer in the world, we reasoned that green tea or EGCG may be an effective agent for chemoprevention of prostate cancer. The present studies are conducted (as a part of Specific Aim I) to investigate the effect of oral consumption of green tea or its major polyphenol, EGCG on prostate tumorigenesis models relevant for human prostate cancer. Employing male TRAMP mice, here we report that oral infusion of a polyphenolic fraction isolated from green tea (GTP), at a human achievable dose significantly inhibits prostate cancer development and increases life span and cancer free survival of these mice.

BODY:

Studies from our laboratory and elsewhere, in cell culture system have shown that green tea and EGCG causes (a) inhibition of cell growth, (b) cell cycle arrest, and (c) induction of apoptosis in several human prostate carcinoma cells (*Paschka et al. Cancer Lett.130: 1-7, 1998 & Gupta et al. Toxicol. App.Pharmacol.164: 82-90, 2000; Gupta et al. Cancer Research 59: 2115-20, 1999*). Further *in vivo* studies from our laboratory have shown that oral feeding of 0.2% GTP in drinking water resulted in a significant decrease in testosterone caused induction of ODC activity in sham-operated and castrated rats, respectively. Similar results were obtained with C57BL/6 mice where oral consumption of GTP resulted in significant inhibition in ODC induction in the ventral prostate (*Gupta et al. Cancer Research 59: 2115-20, 1999*). Extending these studies (Specific aim IA) in animal model we have convincingly shown that green tea at human achievable dose significantly inhibits prostate carcinogenesis in **t**ransgenic **a**denocarcinoma of the **m**ouse **p**rostate (TRAMP) model. In this study we have shown that oral infusion of a polyphenolic fraction isolated from green tea at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibits prostate cancer development and blocks metastases in these mice. In two separate experiments, the cumulative incidence of palpable tumors at 32 weeks of age in 20 untreated mice was 100% (20 of 20). In these mice, 95% (19 of 20), 65% (13 of 20), 40% (8 of 20) and 25% (5 of 20) of the animals exhibited distant site metastases to lymph nodes, lungs, liver and bone, respectively. However, 0.1% GTP (w/v) provided as the sole source of drinking fluid to TRAMP mice from 8 to 32 weeks of age resulted in (a) significant delay in primary tumor incidence and tumor burden as assessed sequentially by MRI, (b) significant decrease in prostate (64%) and genitourinary (72%) weight, (c) significant inhibition in serum IGF-I and restoration of IGFBP-3 levels, and (d) marked reduction in the protein expression of PCNA in the prostate compared to water-fed TRAMP mice. The striking observation of this study was that GTP infusion resulted in almost complete inhibition of distant site metastases. Furthermore, GTP consumption caused significant apoptosis of prostate cancer cells, which possibly resulted in reduced dissemination of cancer cells thereby causing inhibition of prostate cancer development, progression and metastasis of prostate cancer to distant organ sites.

Results:

a) Oral infusion of GTP inhibits prostate tumorigenesis and genito-urinary (GU) weight in TRAMP mice.

To investigate the effect of GTP infusion on prostate cancer growth and progression in TRAMP mice, in two separate experiments, 0.1% GTP was supplied to the animals as the sole source of drinking fluid for 24 weeks starting at the age of 8 weeks. As summarized in table 1 below, in the first experiment, as expected all the ten mice in the water-fed group developed severe prostate cancer with marked local invasiveness in the abdominal region, which was assessed by abdominal pelvic palpation and MRI. In contrast, only three of the ten (30%) GTP-infused TRAMP mice developed palpable tumors. Similarly, in the repeat experiment, all ten

mice in the control group developed fully malignant and palpable tumors whereas in GTP-infused group only four of the ten (40%) animals exhibited palpable tumors. Importantly, in these GTP-infused mice the invasiveness of prostate cancer was much less as compared to water-fed mice. Further, we studied the effect of GTP infusion on the metastases to different site organs. The cumulative data at the termination of the experiment (32 weeks of age) from twenty animals in water-fed group showed 100% invasive tumors, which metastasize to lymph (95% animals), lungs (65% animals), liver (40% animals) and bone (25% animals) respectively. In sharp contrast, in the GTP-infused group, none of the twenty mice exhibited metastases to any of the distant organs studied. Further, to determine the effect of GTP infusion on prostate cancer in TRAMP mice, gross biological indicies (wet weights) were used to assess the tumorigenicity. As observed visibly, GTP infusion resulted in complete absence of hyperplasia in the GU apparatus, especially in the seminal vesicles. An important finding from this experiment was that GTP infusion resulted in a significant decrease in prostate weight (~64%) and GU-weight (~72%) compared to water-fed TRAMP group.

Group[b]	Number of Animals	Palpable Tumor[c]	Animals with Metastasis[d]				Prostate weight (mg)	GU-weight (g)
			Lymph	Lungs	Liver	Bone		
Experiment 1								
Non-TG Control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	17.4 ± 1.4	0.45 ± 0.05
TRAMP water-fed	10	10/10	9/10 (90%)	7/10 (70%)	4/10 (40%)	3/10 (30%)	68.2 ± 8.4	3.76 ± 0.48
TRAMP GTP-fed	10	3/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	32.6 ± 2.8*	1.08 ± 0.12*
Experiment 2								
Non-TG Control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15.6 ± 1.2	0.52 ± 0.04
TRAMP water-fed	10	10/10	10/10 (100%)	6/10 (60%)	4/10 (40%)	2/10 (20%)	83.7 ± 11.6	4.36 ± 0.58
TRAMP GTP-fed	10	4/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	36.2 ± 3.8*	1.20 ± 0.14*
Cumulative								
Non-TG Control	20	0/20	0/10 (0%)	0/20 (0%)	0/10 (0%)	0/10 (0%)	16.5 ± 1.8	0.49 ± 0.05
TRAMP water-fed	20	20/20	19/20 (95%)	13/20 (65%)	8/20 (40%)	5/20 (25%)	76.0 ± 10.8	4.06 ± 0.64
TRAMP GTP-fed	20	7/20	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	27.5 ± 3.2*	1.14 ± 0.16*

[a] The data represented in each experiment is the mean ± SE of 10 mice.
[b] Mice (8 weeks of age) received plain drinking water (control group) or GTP (0.1% w/v) infusion in drinking water for 24 weeks. At the age of 32 weeks, the animals were sacrificed and studied for prostate tumorigenesis and metastases.
[c] Prostate tumor was assessed by abdominal pelvic palpation.
[d] Metastases in the lymph, liver and bone was examined under the microscope while metastasis in lungs was examined by the India ink method. Details are described in 'Materials and Methods'.
* $p < 0.001$, water-fed, control TRAMP compared with GTP-fed TRAMP, Student's 't' test.

b) Oral infusion of GTP inhibits prostate cancer development in TRAMP mice- An MRI analysis. To assess the effect of GTP infusion in TRAMP mice on prostate carcinogenesis we measured the prostate growth using magnetic resonance imaging (MRI).

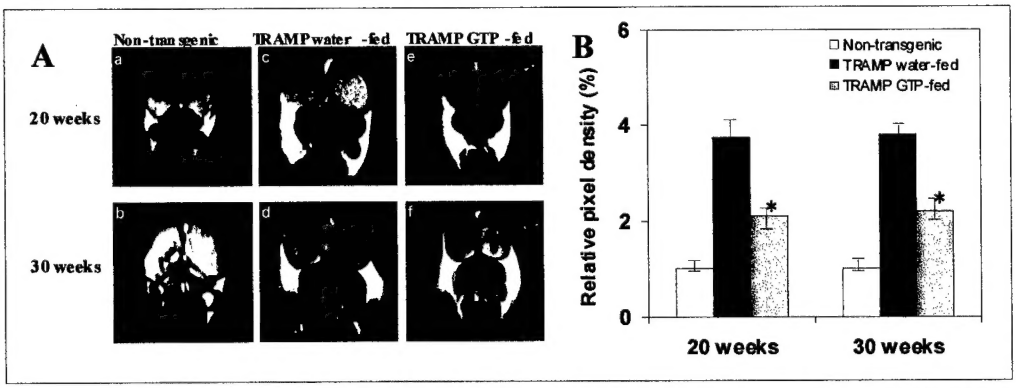


Fig. 1: Effect of GTP infusion on prostate cancer development in TRAMP mice evaluated by longitudinal MRI analysis. **(A)** MRI was used to assess the growth of primary tumor in TRAMP mice followed longitudinally in individual animal. A marked reduction

in prostate development was observed in these mice after 0.1% (w/v) GTP infusion between 8 to 32 weeks. Representative images of **(a, b)** non-transgenic, **(c, d)** water-fed TRAMP mice and **(e, f)** GTP-fed TRAMP mice are shown here at 20 **(a, c, e)** and 30 **(b, d, f)** weeks of age. Arrows indicate prostate. **(B)** Volumetric analysis of the TRAMP mice prostate after GTP infusion. The data is represented as percent change in relative pixel density observed at 20 and 30 weeks of age where non-transgenic mice prostate is taken as control. Values represent mean ± SE of 5 animals. *p <0.001 compared to TRAMP water-fed.

As shown by MRI scans, water-fed TRAMP mice demonstrated the presence of prostate tumor at 20 weeks of age, when the tumor was also detectable by abdominal pelvic palpation (Figure 1A, panel c). As evident by MRI, at 30 weeks the water-fed TRAMP mice were found to have fully developed tumor (Figure 1A, panel d). In sharp contrast, 0.1% GTP infusion to TRAMP mice was found to result in significant prevention or delay in prostate cancer development (Figure 1A, panel e & f). Compared to water-fed TRAMP mice, GTP-infused animals exhibited a marked reduction in the growth of prostate tumor at 20 weeks of age (44% inhibition) and at 30 weeks of age (42% inhibition) respectively, as observed by the volumetric analysis of the prostate (Figure 1B). This was also evident from abdominal pelvic palpation.

c) Oral infusion of GTP decreases serum IGF-1 and restores IGFBP-3 levels in TRAMP mice. In clinical practice, to monitor prostate cancer progression in humans, levels of prostate-specific antigen (PSA), insulin-like growth factor-I (IGF-I), and insulin-like growth factor binding protein-3 (IGFBP-3) in serum are determined. Since in TRAMP mice, like other mice, the murine equivalent of PSA has not yet been identified and/or isolated, we monitored the effect of GTP infusion on growth and development of prostate cancer by determining the levels of IGF-I and IGFBP-3 in serum. We monitored serum IGF-I and IGFBP-3 after 8, 16 and 24 weeks of GTP infusion. For comparison, these levels were also measured in non-transgenic littermates that did not develop prostate cancer. As shown in figure 2A, compared to non-transgenic animals, increasing levels of IGF-I were observed in water-fed TRAMP mice that were significantly lowered in GTP-infused mice. In contrast, serum IGFBP-3 levels, the major binding protein for IGF-I were lower in water-fed TRAMP mice and were significantly restored in GTP-infused mice (Figure 2B).

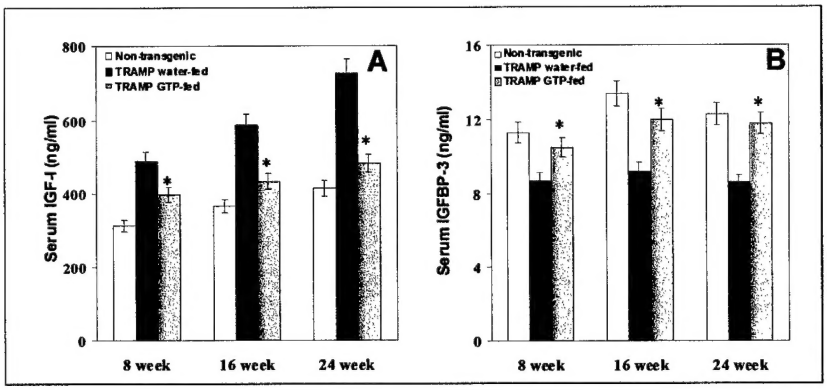


Fig. 2: Effect of GTP infusion on serum levels of **(A)** IGF-I and **(B)** IGFBP-3 in TRAMP mice. Eight weeks old TRAMP mice were infused with 0.1% GTP (w/v) as sole source of drinking fluid for 24 weeks. Blood was withdrawn at 8, 16, and 24 weeks after GTP infusion and serum IGF-I and IGFBP-3 levels were analyzed by enzyme linked immunoabsorbant assay. Details are described in '**Appendix-1**'. A marked inhibition in serum IGF-I and restoration in serum IGFBP-3 levels were observed after GTP infusion. Values represent mean ± SE of 10 animals. *p <0.001 compared to TRAMP water-fed.

d) Oral infusion of GTP decreases proliferation in genito-urinary region and the prostate of TRAMP mice. An important observation of GTP infusion was that it resulted in a significant decrease in GU-weight compared to water-fed TRAMP group (Figure 3, Panel a & b). Further, histological examination of a typical TRAMP mouse prostate tissue at 32 weeks of age revealed prostatic neoplasia characterized by a pronounced proliferation of papillary structures lined by pseudostratified neoplastic cells with marked hyperchromasia and scattered apoptosis. In contrast, the experimental group of GTP-infused mouse exhibited glands lined by uniform columnar cells with dispersed chromatin and minimal luminal infoldings. GTP infusion also resulted in a significant increase in the number of apoptotic cells in the prostate (Figure 3, Panel c & d). GTP infusion for 24 weeks resulted in a marked reduction in PCNA protein expression in the prostate of TRAMP mice

compared with the water-fed group. (Figure 3, Panel B). These results were further confirmed by immunohistochemical analysis of the tissue (Figure 3, Panel B).

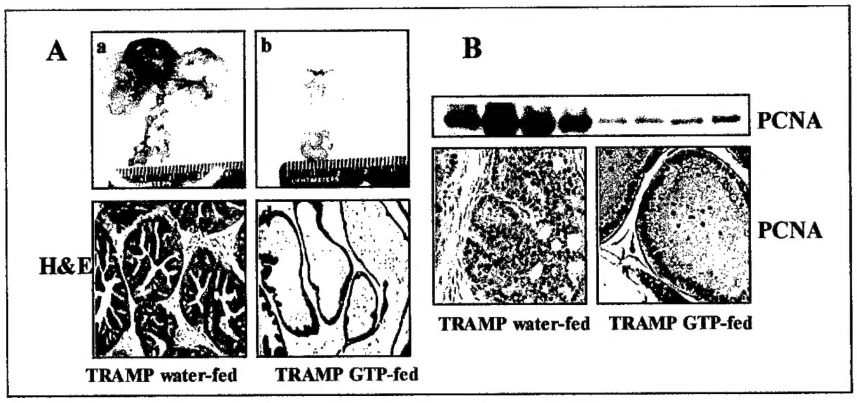


Fig. 3: (A) Effect of GTP infusion on GU-apparatus and prostate histology in TRAMP mice. **(a)** Photograph of typical GU-apparatus of TRAMP mice exhibiting hyper-proliferation, **(b)** GU-apparatus of TRAMP mice with 0.1% GTP infusion (w/v) for 24 weeks. A marked decrease in GU-weight and volume was observed in TRAMP mice after GTP consumption. **(c)** Histologic examination of a typical TRAMP mouse prostate at 32 weeks of age revealed moderately-differentiated neoplastic cells with extensive cribriform structures, marked thickening, remodeling and hypercellularity of the fibromuscular stroma. Magnification=40X **(d)** 0.1% GTP infusion (w/v) to TRAMP mice resulted in a marked reduction in epithelial stratification and cribriform structures with little or no glandular formation in the prostate. Magnification=40X. Representative figures are shown here. **(B)** Effect of GTP infusion on the protein expressions of PCNA in the TRAMP mice prostate. Protein expression of PCNA was determined by Western blot, and immunohistochemical analysis. In water-fed TRAMP, an extensive PCNA staining was observed in the nuclei. 0.1% GTP infusion (w/v) resulted in the marked reduction in the protein expression of PCNA in these mice.

e) Oral infusion of GTP causes apoptosis of tumor cells in the prostate of TRAMP mice. Since green tea is known to induce selective apoptosis in cancer cells, we hypothesized that the observed inhibition of prostate tumorigenesis by GTP infusion is mediated by increased apoptosis of cancerous cells. We first performed ELISA for detection of apoptosis. As shown in figure 4A, GTP infusion for 24 weeks resulted in a significant increase in apoptosis in the prostate of TRAMP mice. In the second approach, these results were further confirmed by immunofluorescence detection in the prostate tissue by M30 CytoDEATH antibody that binds to a caspase cleaved formalin-resistant epitope of the cytokeratin 18 cytoskeletal protein, a marker for apoptosis (Figure 4B). A significant increase in apoptotic index (2.12 $\pm$ 0.1 *versus* 27.7 $\pm$ 3.2% control *versus* GTP-infused) was observed in GTP-infused mice prostate compared with water-fed TRAMP mice.

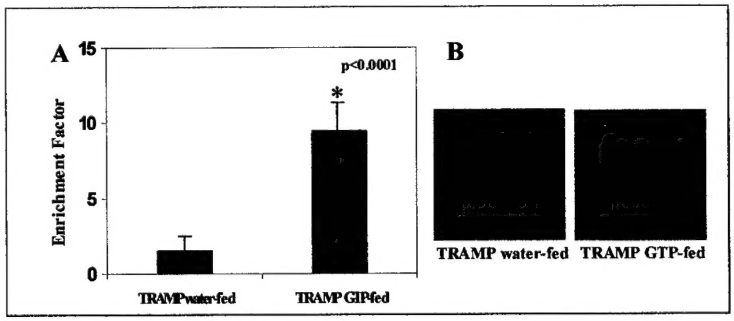


Fig. 4: Effect of GTP infusion on the extent of apoptosis in the TRAMP mice prostate. **(A)** Apoptosis was determined by cell-death ELISA^{PLUS} as per vendor's protocol. Data are expressed as Enrichment factor. Values represent mean $\pm$ SE of 10 animals. *p <0.0001 compared to water-fed TRAMP mice. **(B)** Immunofluorescence detection of prostate tissue in water-fed and GTP-fed TRAMP mice by M30 CytoDEATH antibody, a marker of apoptosis. A marked increase in M30 fluorescence was observed after 0.1% GTP infusion (w/v), compared to water-fed TRAMP mice. A representative figure from each group at 80X magnification is shown here.

f) Oral Infusion of GTP increases Tumor Free Survival and Survival Probability in TRAMP Mice. Since extended tumor free survival and survival probability is the most desirable effect of any chemoprevention regimen therefore, we evaluated whether or not GTP infusion leads to tumor free survival and prolong life expectancy of TRAMP mice. Our data indicated (Figure 5) that continuous GTP infusion to TRAMP mice

actually resulted in the prolongation of the life span of these mice. The continuous GTP infusion to TRAMP mice significantly increased the tumor free survival ($p<0.001$, log-rank test) inasmuch as 50% of the animals remain tumor-free up to 40 weeks of age (Figure 5A). In addition, GTP-fed TRAMP mice exhibited a significant increase (70% higher) in life expectancy ($p<0.001$, log-rank test) with a median survival of 68 weeks compared to the 42 weeks in water-fed TRAMP mice (Figure 5B).

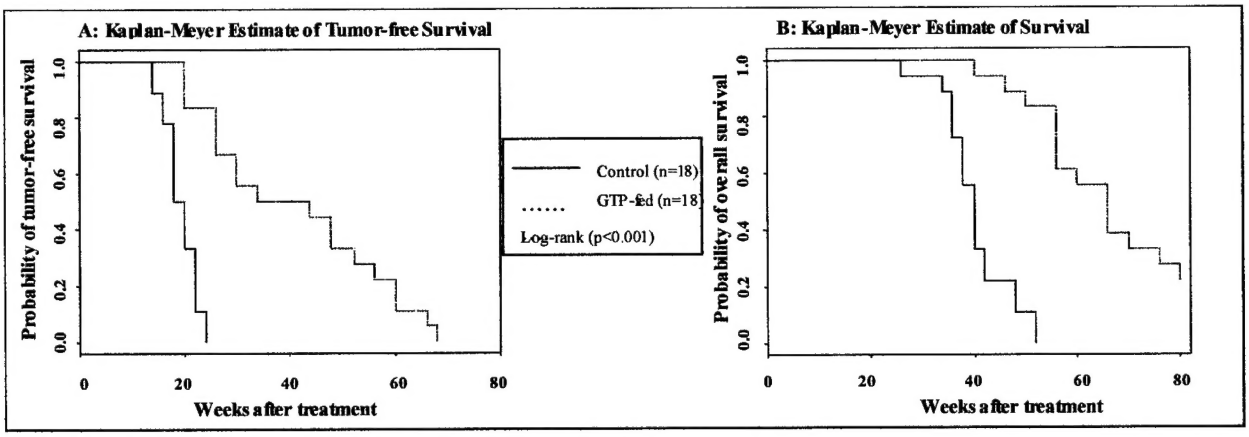


Fig. 11: Effect of GTP infusion on **(A)** Tumor free survival and **(B)** Survival probability in TRAMP mice. A significant increase in tumor free survival ($p<0.001$, log-rank test) and survival probability ($p<0.001$, log-rank test) in GTP-fed TRAMP was observed.

KEY RESEARCH ACCOMPLISHMENTS:

The work presented in the above report for the first time describes that consumption of green tea polyphenols consisting EGCG as major constituent

- **inhibits prostate carcinogenesis in TRAMP mice at physiological achievable dose in humans (equivalent to six cups of green tea per day)**
- **decreases the growth of primary prostate tumor**
- **reverses the serum levels of IGF-1 and restores IGFBP-3**
- **reduction of proliferation in the prostate**
- **complete inhibition of distant site metastases**
- **causes apoptosis in prostate cancer cells**
- **prolongs life and tumor free-survival**

REPORTABLE OUTCOMES:

Abstract # 3386 presented at 91st Annual Meeting of American Association for Cancer Research on April 1-5, 2000 at San Francisco, CA. ***(Appendix-1)***

Chemoprevention of prostate cancer in TRAMP mice by oral infusion of green tea polyphenols

Sanjay Gupta[1], Nihal Ahmad[1], Jonathan S Lewin[2], Norman M Greenberg[3] and Hasan Mukhtar[1]
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106
[3]Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Publication:

Inhibition of Prostate Carcinogenesis in TRAMP Mice by Oral Infusion of Green Tea Polyphenols

Sanjay Gupta[1], Kedar Hastak[1], Nihal Ahmad[1], Jonathan S Lewin[2] and Hasan Mukhtar[1]

Proc. Natl. Acad. Sci. USA. (*In Press*) ***(Appendix-2)***

[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106

CONCLUSIONS:

Limited available options for the treatment of prostate cancer and its increasing incidence have spurred the search for novel preventive approaches for the management of this disease. Chemoprevention by the use of dietary agents or synthetic compounds could be one such strategy that may block the neoplastic inception or delay disease progression (1-5). Since prostate cancer is typically diagnosed in men aged 50 years and older, even a slight delay in the onset and subsequent progression of the disease through the use of chemopreventive agent(s) could have important health benefits (2-5). *The most notable implication of our work is that oral infusion of a human achievable dose of green tea results in significant inhibition in development and progression of prostate cancer along with increased survival in an animal model that emulates human disease.* These data, therefore suggest that green tea consumption may have inhibitory effects on prostate carcinogenesis in humans. This must be investigated in future clinical trials in prostate cancer patients.

Epidemiological studies, though inconclusive, have suggested the protective role of green tea against prostate cancer development (6,7). Recent laboratory studies have indicated that green tea and its polyphenolic constituents impart inhibitory effects on the activities of many enzymatic, metabolic and signaling pathways that have relevance to cancer development and progression (8-13). A number of studies have shown the growth inhibitory effects of green tea against many animal tumor bioassay systems including lung, skin and forestomach (14-16). Studies from our laboratory have shown that green tea polyphenols show promising testosterone-mediated cell growth inhibitory effects and anchorage-independent growth of human prostate carcinoma cells LNCaP *in vitro* as well as GTP-infused Cpb:WU rats and C57BL/6 mice *in vivo* (17). Cell culture studies from this laboratory (18) and elsewhere (19,20) have shown that GTP and epigallocatechin-3-gallate, the major polyphenolic constituent of green tea, inhibit growth of several types of human prostate carcinoma cells. Our studies in cell culture have shown that EGCG was effective in imparting growth inhibition cell cycle desregulation and apoptosis of both androgen-sensitive as well as androgen-insensitive human prostate carcinoma cells (21). Notably, EGCG has been shown to cause growth inhibition and regression of human prostate tumors in athymic nude mice (22).

In the present study, we have employed multiple non-invasive techniques to monitor the chemopreventive potential of GTP for the prevention of prostate cancer. Our first effort was to use the non-invasive technique of MRI for the monitoring of prostate cancer development in these mice. Our studies demonstrate for the first time that MRI may be used as an efficient tool for assessing the effectiveness of a chemopreventive agent against prostate cancer in animals. Next, we found that GTP infusion to TRAMP mice caused significant inhibition of serum IGF-I and restoration of serum IGFBP-3 levels. This is consistent with recent epidemiological studies implicating deregulation of the IGF axis in prostate cancer progression (23,24) and showing that serum IGF-I could be a better predictor of prostate cancer risk than serum PSA (25,26). This is an important observation because recent studies have shown that high circulating levels of IGF-I are associated with increased risk of several common cancers, including those of the breast, prostate, lung and colorectum (27-29). The level of IGF-binding protein (IGFBP-3), a major IGF-I binding protein in serum that, in most situations, suppresses the mitogenic action of IGF-I has been shown to be inversely associated with the risk of these cancers. Taken together, the IGF axis, particularly IGF-I and IGFBP-3, could be developed as endpoint biomarkers for monitoring prostate cancer chemoprevention.

Increased proliferation of prostate cancer cells ultimately results in tumor invasion and metastasis leading to significant mortality in humans (30). Unfortunately, over 60% of the newly diagnosed cases of

CaP develop metastatic forms of the disease (30). In the present study, GTP was found to be effective in completely abolishing distant site metastases and cellular proliferation as shown by the proliferation markers *viz.* PCNA. Another important observation of our study was a marked induction of apoptosis in the prostate by GTP infusion. In recent years, apoptosis has gained much attention as a preferential way of eliminating the unwanted cancerous cells (31-34). Recent studies from our laboratory have shown that GTP selectively induces apoptosis of various human carcinoma cells without affecting the normal cells (18). This observation has been verified from many laboratories worldwide (19,20). Our results show that GTP infusion to TRAMP mice results in massive apoptosis of neoplastic prostatic cells and further suggest that GTP could be an effective agent for a preferential elimination of cancerous and pre-cancerous cells *via* a programmed cell death. Based on our data, we believe that the observed inhibition of prostate tumorigenesis and subsequent metastasis by GTP infusion is caused by selective apoptotic death of cancerous cells however, further studies are needed to substantiate this suggestion. Further, studies with intervention trials i.e. green tea polyphenols and EGCG are capable of restricting tumor growth in established tumor in TRAMP model. Since, consumption of green tea polyphenols resulted in almost complete inhibition of distant site metastases in TRAMP model, further studies on the signaling events that leads to vascularization and angiogenesis are warranted.

Studies indicate that 23% of CaP patients undergoing surgical intervention still show the evidence of disease progression (30). Once the disease becomes hormone refractory, most treatment is palliative and the median life span of these patients is less than 12 months. Therefore, agents that may prolong the survival and quality of life of such patients could have immediate clinical importance. In the present study, GTP infusion to TRAMP mice resulted in a significant increase in tumor free survival and survival probability. Based on these results, we suggest that regular consumption of green tea may prolong life expectancy and quality of life in prostate cancer patients.

In summary our results suggest that a mixture of various polyphenols derived from green tea inhibits the growth and progression of prostate cancer in TRAMP mice. Our data support the epidemiologic reports that green tea may reduce prostate cancer risk in humans. It is important to emphasize that during the course of prostate cancer development and progression the effectiveness of green tea is yet not certain in humans. However, based on the present study it is tempting to suggest that green tea in general and polyphenols present therein may prove to be useful supplement in the prevention or slower progress of prostate cancer in humans. We suggest that these experiments should be undertaken as an extension of this paper.

REFERENCES:

1. Mukhtar H, Ahmad N. Green tea in chemoprevention of cancer. Toxicol Sci. 52: 111-117, 1999.

2. Gupta S, Ahmad N, Mukhtar H. Prostate cancer chemoprevention by green tea. Semin Urol Oncol. 17: 70-76, 1999.

3. Denis L, Morton MS, Griffiths K. Diet and its preventive role in prostatic disease. Eur Urol. 35: 377-387, 1999.

4. Morton MS, Griffiths K, Blacklock N. The preventive role of diet in prostatic disease. Br J Urol. 77: 481-493, 1996.

5. Kelloff GJ, Lieberman R, Steele VE, Boone CW, Lubet RA, Kopelovitch L, Malone WA, Crowell JA, Sigman CC. Chemoprevention of prostate cancer: concepts and strategies. Eur Urol. 35: 342-350, 1999.

6. Bushman JL. Green tea and cancer in humans: a review of the literature. Nutr Cancer. 31: 151-159, 1998.

7. Suganuma M, Okabe S, Sueoka N, Sueoka E, Matsuyama S, Imai K, Nakachi K, Fujiki H. Green tea and cancer chemoprevention. Mutat Res. 428: 339-344, 1999.

8. Menegazzi M, Tedeschi E, Dussin D, Carcereri De Prati A, Cavalieri E, Mariotto S, Suzuki H. Anti-interferon-g action of epigallocatechin-3-gallate mediated by specific inhibition of STAT1 activation. FASEB J. Mar 12 [epub ahead of print] 2001.

9. Nam S, Smith DM, Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. J Biol Chem. 276: 13322-13330, 2001.

10. Jung YD, Kim MS, Shin BA, Chay KO, Ahn BW, Liu W, Bucana CD, Gallick GE, Ellis LM. EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells. Br J Cancer. 84: 844-850, 2001.

11. Garbisa S, Sartor L, Biggin S, Salvato B, Benelli R, Albini A. Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. Cancer. 91: 822-832, 2001.

12. Lin JK, Liang YC, Lin-Shiau SY. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. Biochem Pharmacol. 58: 911-915, 1999.

13. Jankun J, Selman SH, Swiercz R, Skrzypczak-Jankun E. Why drinking green tea could prevent cancer. Nature. 387: 561, 1997.

14. Landau JM, Wang ZY, Yang GY, Ding W, Yang CS. Inhibition of spontaneous formation of lung tumors and rhabdomyosarcomas in A/J mice by black and green tea. Carcinogenesis. 19: 501-507, 1998.

15. Katiyar SK, Agarwal R, Mukhtar H. Protection against malignant conversion of chemically induced benign skin papillomas to squamous cell carcinomas in SENCAR mice by a polyphenolic fraction isolated from green tea. Cancer Res. 53: 5409-5412, 1993.

16. Wang ZY, Agarwal R, Khan WA, Mukhtar H. Protection against benzo[a]pyrene- and N nitrosodiethylamine-induced lung and forestomach tumorigenesis in A/J mice by water extracts of green tea and licorice. Carcinogenesis. 13: 1491-1494, 1992.

17. Gupta S, Ahmad N, Mohan RR, Husain MM, Mukhtar H. Prostate cancer chemoprevention by green tea: in vitro and in vivo inhibition of testosterone-mediated induction of ornithine decarboxylase. Cancer Res. 59: 2115-2120, 1999.

18. Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst. 89: 1881-1886, 1997.

19. Paschka AG, Butler R, Young CY. Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. Cancer Lett. 130: 1-7, 1998.

20. Yang GY, Liao J, Kim K, Yurkow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis. 19: 611-616, 1998.

21. Gupta S, Ahmad N, Nieminen AL, Mukhtar H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. Toxicol Appl Pharmacol. 164: 82-90, 2000.

22. Gupta S, Husain MM, Ahmad N, Hastak K, Marengo SR, Mukhtar H: Antitumor efficacy of green tea constituent epigallocatechin-3-gallate (EGCG) in androgen-dependent and –independent human prostate tumor xenografts in athymic nude mice: A relationship with levels of circulating prostate specific antigen (PSA). Abstract # 3396, 91st Annual Meeting of American Association for Cancer Research, San Francisco, 2000.

23. Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science. 279: 563-566, 1998.

24. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst. 92: 1472-1489, 2000.

25. Harman SM, Metter EJ, Blackman MR, Landis PK, Carter HB. Serum levels of insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-3, and prostate-specific antigen as predictors of clinical prostate cancer. J Clin Endocrinol Metab. 85: 4258-4265, 2000.

26. Djavan B, Bursa B, Seitz C, Soeregi G, Remzi M, Basharkhah A, Wolfram R, Marberger M. Insulin-like growth factor 1 (IGF-1), IGF-1 density, and IGF-1/PSA ratio for prostate cancer detection. Urology. 54: 603-606, 1999.

27. Stattin P, Bylund A, Rinaldi S, Biessy C, Dechaud H, Stenman UH, Egevad L, Riboli E, Hallmans G, Kaaks R. Plasma insulin-like growth factor-I, insulin-like growth factor-binding proteins, and prostate cancer risk: a prospective study. J Natl Cancer Inst. 92: 1910-1917, 2000.

28. Chan JM, Stampfer MJ, Giovannucci E, Ma J, Pollak M. Insulin-like growth factor I (IGF-I), IGF-binding protein-3 and prostate cancer risk: epidemiological studies. Growth Horm IGF Res. S32-33, 2000.

29. Shim M, Cohen P. IGFs and human cancer: implications regarding the risk of growth hormone therapy. Horm Res. 51: 42-51, 1999.

30. Satariano WA, Ragland KE, Van Den Eeden SK. Cause of death in men diagnosed with prostate carcinoma. Cancer 83: 1180-1188, 1998.

31. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature. 411: 342-348, 2001.

32. Gastman BR. Apoptosis and its clinical impact. Head Neck. 23: 409-425, 2001.

33. Galati G, Teng S, Moridani MY, Chan TS, O'Brien PJ. Cancer chemoprevention and apoptosis mechanisms induced by dietary polyphenolics. Drug Metabol Drug Interact. 17: 311-349, 2000.

34. Lin X, Denmeade SR, Isaacs JT. The genetics of programmed (apoptotic) cell death. Cancer Surv. 25: 173-194, 1995.

APPENDICES:

Appendix-1 **Abstract # 3386 presented at 91st Annual Meeting of American Association for Cancer Research on April 1-5, 2000 at San Francisco, CA.**

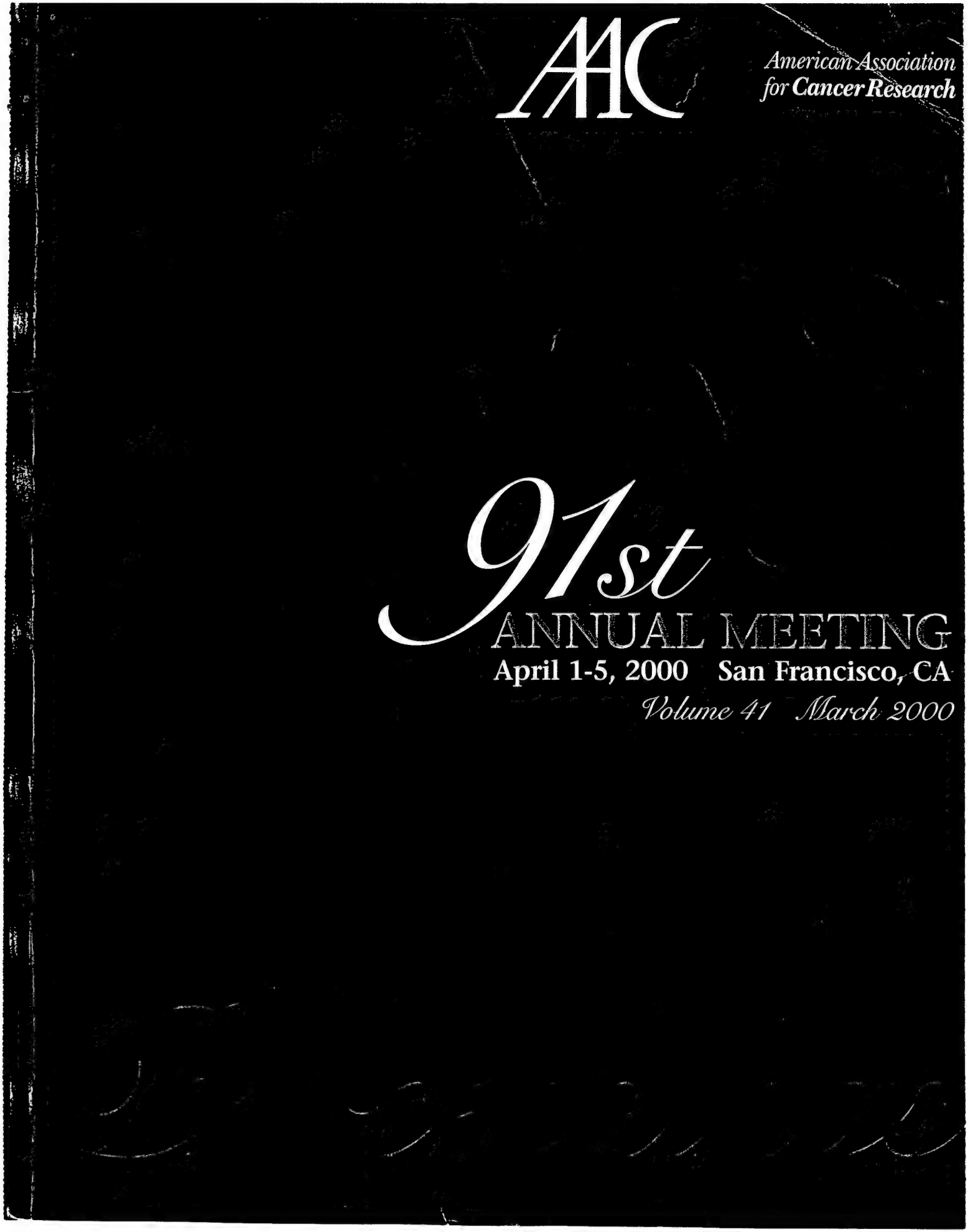
Chemoprevention of prostate cancer in TRAMP mice by oral infusion of green tea polyphenols

Sanjay Gupta[1], Nihal Ahmad[1], Jonathan S Lewin[2], Norman M Greenberg[3] and Hasan Mukhtar[1]
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106
[3]Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Appendix-2 **Publication: In Press**

Inhibition of Prostate Carcinogenesis in TRAMP Mice by Oral Infusion of Green Tea Polyphenols

Sanjay Gupta[1], Kedar Hastak[1], Nihal Ahmad[1], Jonathan S Lewin[2] and Hasan Mukhtar[1]
Proc. Natl. Acad. Sci. USA.
[1]Department of Dermatology, Case Western Reserve University, Cleveland, Ohio 44106
[2]Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106


AACR
American Association for Cancer Research
91st
ANNUAL MEETING
April 1-5, 2000 San Francisco, CA
Volume 41 March 2000

with DNA damage repair following radiation. Immunohistochemical analysis of SCC tumor xenografts shows a reduced expression of VEGF and Factor VIII, suggesting an anti-angiogenic activity of C225. Initial studies examining the capacity of C225 to influence cell invasion/metastasis demonstrate significant inhibition of both basal migration as well as EGF-stimulated migration of fibroblasts using microchemotaxis assays. Preliminary investigation also suggests a reduction in the transverse of SCC cells through extracellular matrix gel following pre-incubation with C225. Taken together, the collective data suggests that the profound antitumor activity of C225 in combination with radiation in SCC model systems may derive from impaired DNA damage repair and decreased angiogenic/metastatic potential in addition to proliferative growth inhibition.

#3382 EXPRESSION OF HER2 IN THE HEART AS A CAUSE FOR THE CARDIOTOXIC EFFECT OF TRASTUZUMAB (HERCEPTIN™)?. Ilka Fuchs, Helmut Buehler, Katja Evers, Sarah Coupland, Uwe Kuehl, and Gerhard Schaller, *Charite Campus Virchow, Humboldt Univ Berlin, Berlin, Germany, and Univ Hosp Benjamin Franklin, Berlin, Germany*

Objective: The therapy of breast cancer with the FDA approved HER2-antibody trastuzumab (Herceptin™) in combination with anthracyclines is very effective, but limited by a dramatic increase of cardiotoxicity. A direct toxic effect of trastuzumab on damaged myocardial cells is an interesting hypothesis, since HER2 plays an essential role during embryonic cardiogenesis. Therefore we investigated myocardial tissue with various histological alterations to detect a supposed upregulation of HER2. Methods: Heart biopsis of 33 patients with clinical manifestations of congestive heart disease or decrease in cardiac systolic function were investigated. Histologically acute myocarditis in 3 cases and chronic myocarditis in 7 cases could be detected. 23 cases showed different degrees of myocardial hypertrophy with various degrees of myocardial fibrosis and necrosis. HER2 expression was analyzed immunhistochemically using the HercepTest™ of DAKO. HER2 gene amplification was analyzed by fluorescence in situ hybridization (FISH) using the Inform-Kit™ of Ventana. Results: None of the described myocardial changes resulted in a HER2 overexpression or gene amplification. Conclusion: Pathological alterations of the heart do not necessarily lead to an expression of HER2 in the myocardium, which could explain the increase in cardiotoxicity during trastuzumab therapy. Further investigations on cardiac tissue treated with anthracylines or trastuzumab are needed to elucidate this question.

#3383 TGF-βRECEPTOR TYPE II RECONSTITUTION SENSITIZES COLON CANCER TO 5-FLUOROURACIL. William M Grady, Ronald M Rerko, and Sanford D Markowitz, *Case Western Reserve Univ /Howard Hughes Med Institute, Cleveland, OH, and Case Western Reserve Univ /University Hosp of Cleveland, Cleveland, OH*

We previously demonstrated mutational inactivation of type II TGF-βreceptors (RII) occurs in 30% of all colon cancers. In light of the frequency of RII mutations in colon cancer and the common clinical use of 5-FU in colon cancer treatment, we investigated the effect of reconstitution of wild-type RII on the sensitivity of colon cancer to 5-FU. Colon cancer cell lines that had mutant RII and that varied in their p53 status (mutant vs. wild-type) and mutation mismatch repair (MMR) status (proficient vs. deficient) were reconstituted with wild-type RII and treated with 5-FU. V-410, a MMR proficient, mutant RII (Arg528His) colon cancer cell line, and HCT116, a MMR deficient, mutant RII cell line (BAT-RII mutation), were reconstituted with RII, treated with 5-FU, and then assessed in a colony forming assay. Reconstitution of RII markedly sensitized both cell lines to 5-FU. V-410RII exhibited a 21-fold reduction in clonal surviving fraction with 5 μM 5-FU compared to the control cell line, V-410Neo. HCT116RII demonstrated a 7-fold reduction in clonal surviving fraction with 3 μM 5-FU compared to the parental line HCT116. The enhancement of 5-FU's effect with RII reconstitution was observed in both V-410 (mutant p53) and HCT116 (wild-type p53) demonstrating it is independent of p53 as well as MMR status. Furthermore, treatment with thymidine, but not uridine, rescued the RII reconstituted cells from 5-FU indicating the interaction between RII and 5-FU involves DNA replication rather than mRNA processing. Thus, reconstitution of wild-type RII in colon cancer enhances 5-FU's effects and appears to operate through a DNA-dependent mechanism. This data suggests a combinatorial therapeutic approach with RII gene therapy and 5-FU could be effective in colon cancer treatment.

#3384 SIGNALING AND PRO-APOPTOTIC CONSEQUENCES OF REGULATED DOMINANT NEGATIVE IGF-I RECEPTOR EXPRESSION. Yasushi Adachi, K. Coffee, S. Nadaf, C. T Lee, and D. P Carbone, *Seoul National Univ Coll of Medicine, Seoul, Korea, Vanderbilt Univ, Nashville, TN, and Vanderbilt-Ingram Cancer Ctr, Nashville, TN*

Continuous growth of tumors depends on the altered regulation of the cell cycle that is in turn modulated by signals from growth factors and their receptors, including IGF-I. Blockade of IGF-IR by antisense or dominant negative plasmid transfection can suppress tumorigenicity and induce regression of established tumors. In order to study the mechanism of this effect and develop potential targeted therapeutics, we have constructed two truncated IGF-IR receptors that function as dominant negative inhibitors of signaling (IGF-IR/dn). These IGF-IR/dn receptors were cloned into recombinant adenoviruses and also placed behind tetracycline regulated promoters for further study. Colon cancer cell lines were stably transfected with IGF-IR/dn engineered to have a stop codon at amino acid residue 486. In the absence of tetracycline, a soluble defective receptor is produced, containing a truncated alpha subunit and lacking the beta subunit, including the transmembrane domain and the tyrosine kinase domain. Soft agar colony assays showed a greater than 1000-fold reduction in the number of colonies in the absence of tetracycline compared with the number in the presence of tetracycline ($p<0.05$). Apoptosis induced by CDDP or 5-FU in addition to steady-state apoptosis were increased 2-3 fold without tetracycline. IGF-IR/dn effectively blocked IGF-IR signal transduction through Akt-1, and IGF-IR/dn activity was detected in the medium of transfectants, suggesting a potentially useful bystander effect. Thus we have demonstrated the combined effectiveness of IGF-IR blockade in combination with chemotherapy and have developed systems that will allow careful study of the mechanisms of the role of IGF-IR in tumor biology. In addition, our adenovirus vectors may have direct potential in cancer gene therapeutics.

PREVENTION/BASIC SCIENCE AND CLINICAL STUDIES 11: Tea Polyphenols

#3385 INHIBITION OF NFκB ACTIVITY BY THEAFLAVIN-3,3′-DIGALLATE FROM BLACK TEA AND OTHER POLYPHENOLS THROUGH DOWN-REGULATING IκB KINASE ACTIVITY IN MACROPHAGES. Jen-Kun Lin, M. H Pan, S. Y Lin-Shiau, and C. T Ho, *National Taiwan Univ, Taipei, Taiwan, and Rutgers Univ, Rutgers, NJ*

IκB kinase (IKK) catalyzes the phosphorylation and degradation of IκB that leads to the activation of NFκB. The inhibition of IKK activity in LPS-activated murine macrophages (RAW 264.7) by several phytopolyphenols including (-)-epigallocatechin-3-gallate, theaflavin, theaflavin-3-gallate, theaflavin-3,3′-digallate (TF-3), pyrocyanidin B-3, casuarinin, geranillin (GA), and penta-O-galloyl-β-D-glucose (5GG) were investigated. TF-3 inhibited IKK activity in LPS-activated macrophages more strongly than other polyphenols. TF-3 strongly inhibited both IKK1 and IKK2 activities, and prevented the degradation of IκBα and IκBβ in the cells. The results suggested that the inhibition of IKK activity by TF-3 could occur by a direct effect on IKKs or on the upstream events in the signal transduction pathways. Furthermore, GA, 5GG and TF-3 all inhibited phosphorylation of IκB from the cytosolic fraction, and also suppressed NFκB activity, and inhibited increases in inducible nitric oxide synthase levels in the activated macrophages. These results suggest that TF-3 and other phytopolyphenols may exert their anti-inflammatory and cancer chemopreventive actions by suppressing the activation of NFκB through inhibition of IKK activity. [This study was supported by the National Science Council and National Health Research Institutes].

#3386 CHEMOPREVENTION OF PROSTATE CANCER IN TRAMP MICE BY ORAL INFUSION OF GREEN TEA POLYPHENOLS. Sanjay Gupta, Nihal Ahmad, Jonathan S Lewin, Norman M Greenberg, and Hasan Mukhtar, *Baylor Coll of Medicine, Dept of Cell Biology, Houstox, TX, Case Western Reserve Univ, Dept of Dermatology, Cleveland, OH, and Univ Hospitals of Cleveland, Dept of Radiology, Cleveland, OH*

Novel chemopreventive approaches are urgently needed for ~40,000 human deaths because of prostate cancer (CaP). To have relevance to human, CaP-chemoprevention studies must be conducted in animal models that emulate human disease, especially where disease progression occurs spontaneously without the administration of unrealistic amounts of chemical carcinogens. Transgenic adenocarcinoma mouse prostate (TRAMP) model spontaneously develops metastatic CaP that mimics human disease. Many studies have demonstrated the cancer chemopreventive effects of a polyphenolic mixture obtained from green tea (GTP). Recently, we showed the antiproliferative effect of GTP in human CaP cells. Employing TRAMP mice, we determined the effect of GTP against CaP development and its subsequent metastasis. In two independent experiments, oral feeding of 0.1% GTP in drinking water for 20 weeks beginning at 8 weeks of age resulted in substantial reduction in tumor burden as assessed sequentially during the course of study by magnetic resonance imaging and at termination of experimennt by measuring size and weight of dorso-lateral prostate and genito-urinary apparatus. Importantly, in GTP-fed mice significant inhibition in serum IGF-1 and restoration of IGFBP-3 was observed at 7, 14 and 20 weeks on test. None out of 20 GTP-fed TRAMP mice exhibited distant site metastases. In sharp contrast, 18 of 20 non-GTP-fed mice exhibited metastases to lymph nodes and lungs. These chemopreventive effects of GTP against CaP development were further confirmed by histopathological examination and proliferation cell nuclear antigen staining in the dorso-lateral prostate. These data demonstrate that green tea could be an effective chemopreventive agent against CaP in humans.

#3387 LACK OF EFFECT OF TOPICAL EPIGALLOCATECHIN GALLATE (EGCG) OINTMENT ON UVB-INDUCED MOUSE SKIN CARCINOGENESIS IN VIVO. Steven P Stratton, Dedun Zhao, Nancy K Hart, David S Alberts, and Robert T Dorr, *Univ of Arizona, Tucson, AZ*

Epigallocatechin gallate (EGCG) is a polyphenol derived from green tea that has been shown to prevent both chemical and UVB-induced skin carcinogenesis in

The Proceedings of the National Academy of Sciences, USA: In Press

Subject Category: **BIOLOGICAL SCIENCES, Medical Sciences**

Inhibition of Prostate Carcinogenesis in TRAMP Mice by Oral Infusion of Green Tea Polyphenols

Sanjay Gupta*, Kedar Hastak*, Nihal Ahmad*, Jonathan S Lewin†, and Hasan Mukhtar*

Affiliations of Authors:

**Department of Dermatology, Case Western Reserve University & The Research Institute of University Hospitals of Cleveland, Cleveland, Ohio 44106*

†Department of Radiology, University Hospitals of Cleveland, Cleveland, Ohio 44106

***Correspondence to:** Hasan Mukhtar, Ph.D.,
Department of Dermatology
Case Western Reserve University
11100 Euclid Avenue
Cleveland, Ohio 44106
Phone: (216) 368 1127
Fax: (216) 368 0212
E-mail: hxm4@po.cwru.edu

Abbreviations: prostate cancer: CaP, green tea polyphenols: GTP, Transgenic adenocarcinoma mouse prostate: TRAMP, probasin: PB, Tag: T-antigen

Key words: Green tea, prostate cancer, chemoprevention, apoptosis, TRAMP

ABSTRACT

Development of effective chemopreventive agents against prostate cancer (CaP) for humans require conclusive evidence of their efficacy in animal models that closely emulate human disease. The autochthonous TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) model, which spontaneously develops metastatic CaP, is one such model that mimics progressive forms of human disease. Employing male TRAMP mice, we show that oral infusion of a polyphenolic fraction isolated from green tea (GTP) at a human achievable dose (equivalent to six cups of green tea per day) significantly inhibits CaP development and increases survival in these mice. In two separate experiments, the cumulative incidence of palpable tumors at 32 weeks of age in 20 untreated mice was 100% (20 of 20). In these mice, 95% (19 of 20), 65% (13 of 20), 40% (8 of 20) and 25% (5 of 20) of the animals exhibited distant site metastases to lymph nodes, lungs, liver and bone, respectively. However, 0.1% GTP (w/v) provided as the sole source of drinking fluid to TRAMP mice from 8 to 32 weeks of age resulted in (a) significant delay in primary tumor incidence and tumor burden as assessed sequentially by MRI, (b) significant decrease in prostate (64%) and genitourinary (72%) weight, (c) significant inhibition in serum IGF-I and restoration of IGFBP-3 levels, and (d) marked reduction in the protein expression of PCNA in the prostate compared to water-fed TRAMP mice. The striking observation of this study was that GTP infusion resulted in almost complete inhibition of distant site metastases. Furthermore, GTP consumption caused significant apoptosis of CaP cells, which possibly resulted in reduced dissemination of cancer cells thereby causing inhibition of prostate cancer development, progression and metastasis of CaP to distant organ sites.

INTRODUCTION

Prostate cancer (CaP) is an important public health problem, accounting for more than 184,000 estimated new cases and approximately 40,000 deaths in the year 2000 alone in the United States (1). In the absence of satisfactory treatment options for CaP, chemoprevention could be an effective approach to reduce the incidence of the disease (2,3). For a variety of reasons there is greater emphasis on identifying naturally occurring dietary substances as cancer chemopreventive agents (3-6). Indeed, CaP is an excellent candidate disease for chemoprevention because it is typically diagnosed in elderly men and therefore even a modest delay in the neoplastic development achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of the clinically detectable disease.

Green tea, a popular beverage consumed worldwide, has been shown to possess cancer chemopreventive effects in a wide range of target organs in rodent carcinogenesis models (4-8). The chemopreventive effects of green tea against tumorigenesis and tumor growth have been attributed to the biochemical and pharmacological activities of its polyphenolic constituents, most notably (-)-epigallocatechin-3-gallate (EGCG), present therein (7-10). Epidemiological studies, though inconclusive, suggest a protective effect of tea consumption on some cancer types in humans (11,12). Limited epidemiological studies indicate that people who consume tea regularly may have a lower risk of CaP (13,14). Further, the Japanese and Chinese populations who regularly consume tea, especially green tea, have one of the lowest incidences of CaP in the world (15,16). In addition, the incidence of CaP is also low in other Asian men, who consume a traditional low-fat diet and tea (15 and references therein).

For relevance to humans, CaP chemoprevention studies should be conducted in animal models that closely emulate human disease and possess surrogate endpoint biomarkers for rapid evaluation of chemopreventive and/or therapeutic agents. Recent developments of

genetically manipulated animals provide new scope for chemoprevention studies and for developing strategies to offset specific genetic susceptibilities to cancer (17,18). The major advantage of these models is that in these animals cancer arises in their natural tissue microenvironment and progress through multiple stages, as does human cancer.

The TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) is one such model for CaP that closely mimics progressive forms of human disease. In this model, expression of the SV40 early genes (T and t antigen, Tag) are driven by the prostate-specific promoter probasin (PB) that leads to cell transformation within the prostate (19). One hundred percent of male TRAMP mice develop CaP without any chemical or hormonal treatment (19,20). Further, CaP in this model progresses from prostatic intraepithelial neoplasia (PIN) to histologic cancer to carcinoma metastatic to lymph nodes, lungs, liver and bone sequentially over 12- 28 weeks with median survival of 42 weeks (20). Recent studies from our laboratory (21) and elsewhere (22,23) have established the utility of these mice for CaP chemoprevention studies. In the present study, we determined the consequence of oral infusion of a polyphenolic fraction isolated from green tea (hereafter referred to as GTP) on CaP development and progression in this model at a human achievable dose. Our results demonstrate that oral infusion of GTP causes a significant inhibition in the development, progression and metastasis of CaP to distant organ sites.

MATERIALS AND METHODS

TRAMP Mice. The male and female TRAMP mice developed on a pure C57BL/6 background, heterozygous for the PB-Tag transgene, were bred and maintained in the Animal Care Facility, School of Medicine, Case Western Reserve University. Transgenic males and the non-transgenic littermates were routinely obtained as [TRAMP C57BL/6 X FVB Breeder] F1. The isolation of mouse-tail DNA and PCR-based screening assay were performed as described

previously (19). All experiments were conducted using the highest standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Study Design for GTP Chemoprevention. GTP (> 95% enriched preparation) was obtained from Natural Resources & Products International Inc. Charlottesville, VA. Chromatographic analysis of this mixture showed that it contains four major polyphenolic constituents: epigallocatechin-3-gallate (62%), epicatechin-3-gallate (24%), epigallocatechin (5%), epicatechin (6%) and caffeine (~1%). The effect of GTP consumption on prostate carcinogenesis in TRAMP mice was studied in two separate experiments. Throughout each experiment, the animals had access to laboratory chow *ad libitum*. For each experiment, 20 male TRAMP mice of 8 weeks of age were equally divided into two groups. A freshly prepared solution of 0.1% GTP in tap water was supplied every Monday, Wednesday and Friday to experimental animals as the sole source of drinking fluid for 24 weeks (GTP-infused group), while the control group of animals was supplied with the same tap water throughout the experiment (water-fed group). This feeding regimen has been used in mice in many prior chemoprevention studies from this and other laboratories (24,25). The feeding protocol mimics an approximate consumption of six cups of green tea per day by an average adult human (25). Additional untreated and treated non-transgenic controls were also included in the study. After completion of the experiment, the animals from both experimental and control groups were sacrificed by cervical dislocation and the prostate gland was carefully removed under the microscope for further studies.

To investigate the effect of GTP consumption on tumor free survival, in a third experiment, 36 male mice of 8 weeks of age were equally divided into two groups. The control group of animals were supplied with tap water while animals in the experimental group were infused with 0.1% GTP (w/v) in drinking water exactly as in the first two experiments. Animals of both groups were monitored biweekly for tumor development by abdominal pelvic palpation and

survival. For these studies, the animals were sacrificed by CO_2 asphyxiation when obviously moribund.

Magnetic Resonance Imaging (MRI). Five animals each from both experimental and control groups were randomly selected and monitored for tumor growth and volume by MRI at 20 and 30 weeks of age. Imaging in these animals was performed using a whole body 1.5 Telsa imager with 25 mT/m gradient strength, 150 microsecond rise time, and a custom built 1 cm small animal receiver coil. T1-weighted (TR/TE=400 ms/14 ms), double echo T2-weighted (TR/TE=1900 ms/20, 84 ms), and CISS T2-weighted (TR/TE/Flip angle=12.3 ms/5.9 ms/70°) gradient echo volumetric scans with a field of view of between 2 and 5 cm and in plane resolution of 78-200 micron were obtained with a slice thickness of 500-2000 microns. Images were filmed for subjective analysis and/or transferred to a free-standing imaging workstation for volumetric analysis of prostate tumor.

IGF-1 and IGFBP-3 Assay. Serum was separated from the whole blood obtained from the retro-orbital venous plexus with heparinized capillary tubes and IGF-1 and IGFBP-3 levels were determined by commercially available ELISA kits (Diagnostic Systems Laboratories, Inc. Webster, TX) according to the manufacturer's protocol. The sensitivity of the assay was 0.04 ng/ml and virtually no cross-reactivity was visible with other members of the group.

Preparation and Analysis of Tissue. At the time of sacrifice, the lower GU tract, including the bladder, testes, seminal vesicles, and prostate, were removed *en bloc*. The GU wet weight was recorded to the nearest 0.01 g. Tissues collected at necropsy were fixed in 10% (v/v) phosphate-buffered formalin for 12 hrs and then transferred to 70% ethanol prior to standard tissue processing. Sections of the prostate (4 μm) were cut from paraffin-embedded tissues and mounted on ProbeOn-Plus slides (Fisher Scientific, Houston, TX). Sections were stained with H&E and were reviewed by light microscopy for the presence of CaP. For the metastasis examination in the lymph and liver, tissues from all the animals were examined under the

microscope whereas for lung metastasis the India ink method was used as described previously (21). For analysis of possible bone metastasis, bone tissue including forelimbs, hind limbs, pelvis, thoracic and lumbar vertebrae was fixed in 10% (v/v) phosphate-buffered formalin for 48 h, decalcified in 14% EDTA for two weeks, processed through graded alcohols, and embedded in paraffin wax. Sections were stained with orange G and phloxine, and were analyzed under the microscope.

Immunoblotting and Immunohistochemistry. Prostate glands were removed from the animals and homogenized in lysis buffer (50 mM Tris-HCl, 150 nM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4) at 4^0 C to prepare cell lysates. Appropriate amount of protein (25-50 μg) was resolved over 12%-Tris-Glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% non-fat dry milk and probed using appropriate primary antibodies for PCNA, SV 40 T-antigen (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer for 1 hour to overnight at 4^0 C. The membrane was then incubated with appropriate secondary antibody horseradish peroxidase (HRP) conjugate (Amersham Life Sciences Inc., Arlington Heights, IL) followed by detection using chemiluminescence ECL kit (Amersham Life Sciences Inc., Arlington Heights, IL). For confirmation of equal loading the membrane was stripped and reprobed with β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), a housekeeping protein and appropriate secondary HRP conjugate.

For immunohistochemical analysis the sections (4 μm) were cut from paraffin-embedded tissues. Immuno-staining was performed using antibody for PCNA with appropriate dilutions which was replaced with either normal host serum or block for negative controls, followed by counter staining with weak hematoxylin stain. The stained slides were examined under the microscope.

Image Analysis. Sections were visualized on a Zeiss-Axiophot DM HT microscope. Images were captured with an attached camera linked to a computer. Images and figures were composed using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

Immunofluorescence Analysis and Apoptosis Detection. Four-micrometer thick sections were cut from paraffin-embedded tissues. Immunofluorescence was performed using M30 CytoDEATH antibody (Boehringer Mannheim GmbH) with a fluorescence microscope (model Axiophot, Carl Zeiss, Inc.). Scoring of apoptotic cells in these sections was done using the Optimas 6 software program (Optimas Corp., Bothell, WA). Apoptotic index (%) was calculated by dividing the number of apoptotic cells (fluorescence positive) by the total number of cells counted per cross-section of a sample of the prostate.

Apoptosis by ELISA. Apoptosis was also assessed by Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. This method is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histone fragments, which are hallmarks of apoptosis. The values are calculated as 'Enrichment factor', which is the specific enrichment of mono- and oligonucleosomes released into the cytoplasm (absorbance values) of the experimental group divided by the corresponding control.

Statistical Analysis. All statistical analyses were carried out with Statistical Analysis System software (Cary, NC) and p values less than 0.05 were considered significant. The Kaplan-Meier method was used to estimate survival and differences were analyzed by the log-rank test.

RESULTS

MRI Analysis of TRAMP Mice Infused with GTP. To assess the effect of GTP infusion in TRAMP mice on prostate carcinogenesis we first measured the prostate growth using magnetic resonance imaging (MRI). MRI is considered a powerful tool for imaging internal organs and for diagnosis of certain cancer types in humans (26). We employed this technique for monitoring

the effect of GTP infusion on CaP development and progression in TRAMP mice (Figure 1A). As shown by MRI scans, water-fed TRAMP mice demonstrated the presence of prostate tumor at 20 weeks of age, when the tumor was also detectable by abdominal pelvic palpation (Figure 1A, panel c). As evident by MRI, at 30 weeks the water-fed TRAMP mice were found to have fully developed tumor (Figure 1A, panel d). In sharp contrast, 0.1% GTP infusion to TRAMP mice was found to result in significant prevention or delay in prostate cancer development (Figure 1A, panel e & f). Compared to water-fed TRAMP mice, GTP-infused animals exhibited a marked reduction in the growth of prostate tumor at 20 weeks of age (44% inhibition) and at 30 weeks of age (42% inhibition) respectively, as observed by the volumetric analysis of the prostate (Figure 1B). This was also evident from abdominal pelvic palpation.

Effect of GTP Infusion on Serum IGF-1 and IGFBP-3 Levels. In clinical practice, to monitor CaP progression in humans, levels of prostate-specific antigen (PSA), insulin-like growth factor-I (IGF-I), and insulin-like growth factor binding protein-3 (IGFBP-3) in serum are determined. Since in TRAMP mice, like other mice, the murine equivalent of PSA has not yet been identified and/or isolated, we monitored the effect of GTP infusion on growth and development of CaP by determining the levels of IGF-I and IGFBP-3 in serum. Recent studies have demonstrated that elevated levels of IGF-I with concomitant lowering of IGFBP-3 levels in serum is associated with CaP risk and could be excellent predictors of CaP progression in humans (27). We monitored serum IGF-I and IGFBP-3 after 8, 16 and 24 weeks of GTP infusion. For comparison, these levels were also measured in non-transgenic littermates that did not develop CaP. As shown in figure 2A, compared to non-transgenic animals, increasing levels of IGF-I were observed in water-fed TRAMP mice that were significantly lowered in GTP-infused mice. In contrast, serum IGFBP-3 levels, the major binding protein for IGF-I were lower in water-fed TRAMP mice and were significantly restored in GTP-infused mice (Figure 2B).

Effect of GTP Infusion on Prostate Tumorigenesis. GTP infusion for 24 weeks to TRAMP mice did not exhibit any symptoms of toxicity or apparent signs of ill health. No significant affect

was observed in the body weight profile in non-transgenic littermates infused with 0.1% GTP when compared to the water-fed non-transgenic controls. However, TRAMP mice receiving GTP infusion registered a slight decrease in body weight (~5%) compared to their corresponding control group (data not shown). This difference may be due to more tumor growth and hyperproliferation of the accessory sex organs in the abdominal region that occurs in control TRAMP mice.

To investigate the effect of GTP infusion on CaP growth and progression in TRAMP mice, in two separate experiments, 0.1% GTP was supplied to the animals as the sole source of drinking fluid for 24 weeks starting at the age of 8 weeks. As summarized in table 1, in the first experiment, as expected all the ten mice in the water-fed group developed severe CaP with marked local invasiveness in the abdominal region, which was assessed by abdominal pelvic palpation and MRI. In contrast, only three of the ten (30%) GTP-infused TRAMP mice developed palpable tumors. Similarly, in the repeat experiment, all ten mice in the control group developed fully malignant and palpable tumors whereas in GTP-infused group only four of the ten (40%) animals exhibited palpable tumors. Importantly, in these GTP-infused mice the invasiveness of CaP was much less as compared to water-fed mice. Further, we studied the effect of GTP infusion on the metastases to different site organs. The cumulative data at the termination of the experiment (32 weeks of age) from twenty animals in water-fed group showed 100% invasive tumors, which metastasize to lymph (95% animals), lungs (65% animals), liver (40% animals) and bone (25% animals) respectively. In sharp contrast, in the GTP-infused group, none of the twenty mice exhibited metastases to any of the distant organs studied. Further, to determine the effect of GTP infusion on CaP in TRAMP mice, gross biological indicies (wet weights) were used to assess the tumorigenicity (Table 1). As observed visibly, GTP infusion resulted in complete absence of hyperplasia in the GU apparatus, especially in the seminal vesicles. An important observation in this experiment was that GTP infusion resulted in

a significant decrease in prostate weight (~64%) and GU-weight (~72%) compared to water-fed TRAMP group (Table 1; Figure 3A & B).

Effect of GTP Infusion on Prostate Histology. Histological examination of a typical TRAMP mouse prostate tissue at 32 weeks of age revealed prostatic neoplasia characterized by a pronounced proliferation of papillary structures lined by pseudostratified neoplastic cells with marked hyperchromasia and scattered apoptosis. In contrast, the experimental group of GTP-infused mouse exhibited glands lined by uniform columnar cells with dispersed chromatin and minimal luminal infoldings. GTP infusion also resulted in a significant increase in the number of apoptotic cells in the prostate (Figure 3C & D).

Effect of GTP Infusion on Proliferation Marker. We next determined the effect of GTP infusion on cellular proliferation in prostate as assessed by following the ubiquitous and molecular proliferation marker PCNA. PCNA serves as a requisite auxiliary protein for DNA polymerase δ-driven DNA synthesis and is cell cycle regulated (28,29). GTP infusion for 24 weeks resulted in a marked reduction in PCNA protein expression in the prostate of TRAMP mice compared with the water-fed group. (Figure 4A). These results were further confirmed by immunohistochemical analysis of the tissue (Figure 4B). Further, the effect of GTP infusion on Tag expression (T-antigen) was determined in prostates of TRAMP mice. GTP infusion did not result in any significant alteration in the levels of Tag protein expression and they are detectable in both GTP-infused and water-fed groups (data not shown).

Effect of GTP Infusion on the Extent of Apoptosis. Since green tea is known to induce selective apoptosis in cancer cells (30), we hypothesized that the observed inhibition of prostate tumorigenesis by GTP infusion is mediated by increased apoptosis of cancerous cells. To test our hypothesis, we employed multiple approaches of apoptosis determination. In our first approach, ELISA was performed for detection of apoptosis. As shown in figure 5A, GTP infusion for 24 weeks resulted in a significant increase in apoptosis in the prostate of TRAMP mice. In the second approach, these results were further confirmed by immunofluorescence detection in

the prostate tissue by M30 CytoDEATH antibody that binds to a caspase cleaved formalin-resistant epitope of the cytokeratin 18 cytoskeletal protein, a marker for apoptosis (Figure 5B). A significant increase in apoptotic index (2.12 $\pm$ 0.1 *versus* 27.7 $\pm$ 3.2% control *versus* GTP-infused) was observed in GTP-infused mice prostate compared with water-fed TRAMP mice.

Effect of GTP Infusion on Tumor Free Survival and Survival Probability in TRAMP Mice. Extended tumor free survival and survival probability is the most desirable effect of any chemoprevention regimen. Therefore, in the next series of experiment we evaluated whether or not GTP infusion leads to tumor free survival and prolong life expectancy of TRAMP mice. Our data indicated (Figure 6) that continuous GTP infusion to TRAMP mice actually resulted in the prolongation of the life span of these mice. The continuous GTP infusion to TRAMP mice significantly increased the tumor free survival ($p<0.001$, log-rank test) inasmuch as 50% of the animals remain tumor-free up to 40 weeks of age (Figure 6A). In addition, GTP-infused TRAMP mice exhibited a significant increase (70% higher) in life expectancy ($p<0.001$, log-rank test) with a median survival of 68 weeks compared to the 42 weeks in water-fed TRAMP mice (Figure 6B).

DISCUSSION

Limited available options for the treatment of CaP and its increasing incidence have spurred the search for novel preventive approaches for the management of this disease. Chemoprevention by the use of dietary agents or synthetic compounds could be one such strategy that may block the neoplastic inception or delay disease progression (4-6). Since CaP is typically diagnosed in men aged 50 years and older, even a slight delay in the onset and subsequent progression of the disease through the use of chemopreventive agent(s) could have important health benefits. Ideally, the efficacy of such chemopreventive agents should be verified in animal models that emulate human disease before recommending their use for humans. The most notable implication of our work is that oral infusion of a human achievable

dose of green tea results in significant inhibition in development and progression of CaP along with increased survival in an animal model that emulates human disease. These data, therefore suggest that green tea consumption may have inhibitory effects on prostate carcinogenesis in humans.

Epidemiological studies, though not conclusive, have suggested the protective role of green tea against CaP development (10-14). Recent laboratory studies have indicated that green tea and its polyphenolic constituents impart inhibitory effects on the activities of many enzymatic, metabolic and signaling pathways that have relevance to cancer development and progression (31-36). A number of studies have shown the growth inhibitory effects of green tea against many animal tumor bioassay systems including lung, skin and forestomach (37-40). Studies from our laboratory have shown that green tea polyphenols show promising testosterone-mediated cell growth inhibitory effects and anchorage-independent growth of human prostate carcinoma cells LNCaP *in vitro* as well as GTP-infused Cpb:WU rats and C57BL/6 mice *in vivo* (41). Cell culture studies from this laboratory (42) and elsewhere (43-45) have shown that GTP and epigallocatechin-3-gallate, the major polyphenolic constituent of green tea, inhibit growth of several types of human CaP cells. Our studies in cell culture have shown that EGCG was effective in imparting growth inhibition cell cycle desregulation and apoptosis of both androgen-sensitive as well as androgen-insensitive human CaP cells (46). Notably, EGCG has been shown to cause growth inhibition and regression of human prostate tumors in athymic nude mice (47). No prior *in vivo* study has ascertained the effect of green tea infusion on CaP chemoprevention in a prostate carcinogenesis model, partly due to the lack of appropriate animal model that could mimic the progressive forms of human prostatic disease. TRAMP model possess similarity to human disease in the development and progression to metastatic CaP. Recent studies from our laboratory and elsewhere have established the utility of TRAMP mice for CaP chemoprevention studies (21-23). In the present study, we assessed the chemopreventive potential of GTP against prostate carcinogenesis in TRAMP model. Our

results suggest that GTP infusion was significantly effective in inhibiting CaP development and completely abolished distant site metastases. Prior published studies have shown that polyphenols present in green tea and caffeine possess cancer chemopreventive effects (48). Although the role played by caffeine in observed CaP chemopreventive effects could not be ruled out, we believe that the observed effects in this study may be due to the polyphenolic constituents rather than caffeine because of its presence in low concentration (~1%) in the GTP mixture.

In the present study, we have employed multiple non-invasive techniques to monitor the chemopreventive potential of GTP for the prevention of CaP. Our first effort was to use the non-invasive technique of MRI for the monitoring of CaP development in these mice. Our studies demonstrate for the first time that MRI may be used as an efficient tool for assessing the effectiveness of a chemopreventive agent against CaP in animals. Next, we found that GTP infusion to TRAMP mice caused significant inhibition of serum IGF-I and restoration of serum IGFBP-3 levels. This is consistent with recent epidemiological studies implicating deregulation of the IGF axis in CaP progression (49) and showing that serum IGF-I could be a better predictor of CaP risk than serum PSA (50). Our results are also consistent with the previous observation where prostate-specific IGF-I was found to be increased during prostate cancer progression in TRAMP mice (51). This is an important observation because recent studies have shown that high circulating levels of IGF-I are associated with increased risk of several common cancers, including those of the breast, prostate, lung and colorectum (49). The level of IGF-binding protein (IGFBP-3), a major IGF-I binding protein in serum that, in most situations, suppresses the mitogenic action of IGF-I has been shown to be inversely associated with the risk of these cancers. Taken together, the IGF axis, particularly IGF-I and IGFBP-3, could be developed as endpoint biomarkers for monitoring CaP chemoprevention.

Increased proliferation of CaP cells ultimately results in tumor invasion and metastasis leading to significant mortality in humans (52). Unfortunately, over 60% of the newly diagnosed

cases of CaP develop metastatic forms of the disease (53). In the present study, GTP was found to be effective in completely abolishing distant site metastases and cellular proliferation as shown by the proliferation markers *viz*. PCNA. An important observation of the study is that GTP infusion to TRAMP mice did not alter the expression of t/T-antigen as they are readily detectable in both GTP-infused and water-fed groups. These results confirm that mechanism of CaP inhibition by GTP infusion was not through down-regulation of the transgene.

Another important observation of our study was a marked induction of apoptosis in the prostate by GTP infusion. In recent years, apoptosis has gained much attention as a preferential way of eliminating the unwanted cancerous cells (42-46,53). At present, only few agents are known to possess the potential for selective elimination of cancer cells (54 and references therein). Recent studies from our laboratory have shown that GTP selectively induces apoptosis of various human carcinoma cells without affecting the normal cells (42). This observation has been verified from many laboratories worldwide (43-46). Our results show that GTP infusion to TRAMP mice results in massive apoptosis of neoplastic prostatic cells and further suggest that GTP could be an effective agent for a preferential elimination of cancerous and pre-cancerous cells *via* a programmed cell death. Based on our data, we believe that the observed inhibition of CaP tumorigenesis and subsequent metastasis by GTP infusion is caused by selective apoptotic death of cancerous cells however, further studies are needed to substantiate this suggestion.

Studies indicate that 23% of CaP patients undergoing surgical intervention still show the evidence of disease progression (52). Once the disease becomes hormone refractory, most treatment is palliative and the median life span of these patients is less than 12 months. Therefore, agents that may prolong the survival and quality of life of such patients could have immediate clinical importance. In the present study, GTP infusion to TRAMP mice resulted in a significant increase in tumor free survival and survival probability. Based on these results, we

suggest that regular consumption of green tea may prolong life expectancy and quality of life in CaP patients.

In summary our results suggest that GTP, a mixture of various polyphenols inhibit the growth and progression of CaP in TRAMP mice. Our data support the epidemiologic reports that green tea may reduce CaP risk in humans. It is important to emphasize that during the course of CaP development and progression the effectiveness of green tea is yet not certain in humans. However, based on the present study it is tempting to suggest that green tea in general and polyphenols present therein may prove to be useful supplement in the prevention or slower progress of CaP in humans.

ACKNOWLEDGEMENT

Supported by grants from United States Public Health Service (RO1CA 78809), American Institute for Cancer Research (00A030), and the Department of Defense (DAMD 17-00-1-0527) (to H.M.) and by funds from the O-CHA (Tea) Pioneer Academic Research Grant Program, Japan (to S.G.), Cancer Research Foundation of America (to S.G. and N.A.). We acknowledge the assistance of Dr. J. Sunil Rao, Department of Epidemiology and Biostatistics for statistical analysis of data. We are grateful to Norman M. Greenberg, Ph.D. (Baylor College of Medicine, Houston, TX) for providing TRAMP mice and careful reading of the manuscript and to Gregory T. MacLennan, M.D. (University Hospitals of Cleveland, Cleveland, OH) for his expert opinion as a prostate pathologist.

REFERENCES

1. Greenlee, R.T., Murray, T., Bolden, S., & Wingo, P.A. (2000) C.A. Cancer J. Clin. **50,** 7-33.

2. Kamat, A.M., & Lamm, D.L. (1999) J. Urol. **161**, 1748-1746.

3. Boyle, P., & Severi, G. (1999) Eur. Urol. **35**, 370-376.

4. El-Bayoumy, K., Chung, F.L., Richie, J. Jr., Reddy, B.S., Cohen, L., Weisburger, J., & Wynder, E.L. (1997) Proc. Soc. Exp. Biol. Med. **216**, 211-223.

5. Kelloff, G.J., Lieberman, R., Steele, V.E., Boone, C.W., Lubet, R.A., Kopelovitch, L., Malone, W.A., Crowell, J.A., & Sigman, C.C. (1999) Eur. Urol. **35**, 342-350.

6. Safe, S., Wargovich, M.J., Lamartiniere, C.A., & Mukhtar, H. (1999) Toxicol. Sci. **52**, 1-8.

7. Yang, C.S., Chung, J.Y., Yang, G., Chhabra, S.K., & Lee, M.J. (2000) J. Nutr. **130**, 472S-478S.

8. Conney, A.H., Lu, Y., Lou, Y., Xie, J., & Huang, M. (1999) Proc. Soc. Exp. Biol. Med. **220**, 229-233.

9. Weisburger, J.H. (1999) Proc. Soc. Exp. Biol. Med. **220**, 271-275.

10. Katiyar, S.K. & Mukhtar, H. (1996) Int. J. Oncol. **8,** 221-238.

11. Kohlmeier, L., Weterings, K.G., Steck, S., & Kok, F.J. (1997) Nutr. Cancer. **27**, 1-13.

12. Bushman, J.L. (1998) Nutr. Cancer. **31**, 151-159.

13. Heilbrun, L.K., Nomura, A., & Stemmermann, G.N. (1986) Br. J. Cancer. **54**, 677-683.

14. Kinlen, L.J., Willows, A.N., Goldblatt, P., & Yudkin, J. (1988) Br. J. Cancer. **58**, 397-401.

15. Denis, L., Morton, M.S., & Griffiths, K. (1999) Eur. Urol. **35**, 377-387.

16. Gupta, S., Ahmad, N., & Mukhtar, H. (1999) Semin. Urol. Oncol. **17**, 70-76.

17. Alexander, J. (2000) Toxicol. Lett. **112-113**, 507-512.

18. Hursting, S.D., Slaga, T.J., Fischer, S.M., DiGiovanni, J., & Phang, J.M. (1999) J. Natl. Cancer Inst. **91**, 215-225.

19. Greenberg, N.M., DeMayo, F., Finegold, M.J., Medina, D., Tilley, W.D., Aspinall, J.O., Cunha, G.R., Donjacour, A.A., Matusik, .RJ. et al. (1995) Proc. Natl. Acad. Sci. USA. **92**, 3439-3443.

20. Gingrich, J.R., Barrios, R.J., Morton, R.A., Boyce, B.F., DeMayo, F.J., Finegold, M.J., Angelopoulou, R., Rosen, J.M., & Greenberg, N.M. (1996) Cancer Res. **56**, 4096-4102.

21. Gupta, S., Ahmad, N., Marengo, S.R., MacLennan, G.T., Greenberg, N.M., & Mukhtar, H. (2000) Cancer Res. **60**, 5125-5133.

22. Wechter, W.J., Leipold, D.D., Murray, E.D. Jr., Quiggle, D., McCracken, J.D., Barrios, R.S., & Greenberg, N.M. (2000) Cancer Res. **60**, 2203-2208.

23. Raghow, S., Kuliyev, E., Steakley, M., Greenberg, N., & Steiner, M.S. (2000) Cancer Res. **60**, 4093-4097.

24. Liu, Q., Wang, Y., Crist, K.A., Wang, Z.Y., Lou, Y.R., Huang, M.T., Conney, A.H., & You, M. (1998) Carcinogenesis. **19**, 1257-1262.

25. Wang, Z.Y., Agarwal, R., Bickers, D.R., & Mukhtar, H. (1991) Carcinogenesis. **12**, 1527-1530.

26. Gedroyc, W.M. (2000) B.J.U. Int. **86**, 174-180.

27. Chan, J.M., Stampfer, M.J., Giovannucci, E., Gann, P.H., Ma, J., Wilkinson, P., Hennekens, C.H., & Pollak, M. (1998) Science. **279**, 563-566.

28. Prosperi, E. (1997) Cell Prog. Cycle Res. **3**, 193-210.

29. Kelman Z. (1997) Oncogene. **14**, 629-640.

30. Ahmad, N., Gupta, S., & Mukhtar, H. (2000) Arch. Biochem. Biophys. **376**, 338-346.

31. Liao, S., & Hiipakka, R.A. (1995) Biochem. Biophys. Res. Commun. **214**, 833-838.

32. Jankun, J., Selman, S.H., Swiercz, R., & Skrzypczak-Jankun, E. (1997) Nature. **387**, 561.

33. Cao, Y., & Cao, R. (1999) Nature. **398**, 381.

34. Garbisa, S., Biggin, S., Cavallarin, N., Sartor, L., Benelli, R., & Albini, A. (1999) Nat. Med. **5**, 1216.

35. Nam, S., Smith, D.M., Dou, Q.P. (2001) J Biol Chem. **276**, 13322-13330.

36. Menegazzi, M., Tedeschi, E., Dussin, D., Carcereri De Prati, A., Cavalieri, E., Mariotto, S., Suzuki, H. (2001) FASEB J. **15**, 1309-1311.

37. Katiyar, S.K., Agarwal, R., Wood, G.S., & Mukhtar, H. Cancer Res. (1992) **52**, 6890-5897.

38. Katiyar, S.K., Agarwal, R., Zaim, M.T., & Mukhtar, H. (1993) Carcinogenesis. **14**, 849-855.

39. Lu, Y.P., Lou, Y.R., Xie, J.G., Yen, P., Huang, M.T., & Conney, A.H. (1997) Carcinogenesis. **18**, 2163-2169.

40. Landau, J.M., Wang, Z.Y., Yang, G.Y., Ding, W., & Yang, C.S. (1998) Carcinogenesis. **19**, 501-507.

41. Gupta, S., Ahmad, N., Mohan, R.R., Husain, M.M., & Mukhtar, H. (1999) Cancer Res. **59**, 2115-2120.

42. Ahmad, N., Feyes, D.K., Nieminen, A.L., Agarwal, R., & Mukhtar, H. (1997) J. Natl. Cancer Inst. **89**, 1881-1886.

43. Yang, G.Y., Liao, J., Kim, K., Yurkow, E.J., & Yang, C.S. (1998) Carcinogenesis. **19**, 611-616.

44. Valcic, S., Timmermann, B.N., Alberts, D.S., Wachter, G.A., Krutzsch, M., Wymer, J., & Guillen, J.M. (1996) Anticancer Drugs. **7**, 461-468.

45. Paschka, A.G., Butler, R. & Young, C.Y. (1998) Cancer Lett. **130**, 1-7.

46. Gupta, S., Ahmad, N., Nieminen, A.L., & Mukhtar, H. (2000) Toxicol. Appl. Pharmacol. **164**, 82-90.

47. Liao, S., Umekita, Y., Guo, J., Kokontis, J.M., & Hiipakka, R.A. (1995) Cancer Lett. **96**, 239-243.

48. Huang, M.T., Xie, J.G., Wang, Z.Y., Ho, C.T., Lou, Y.R., Wang, C.X., Hard, G.C., Conney, A.H. (1997) Cancer Res. **57**, 2623-2629.

49. Yu, H., & Rohan, T. (2000) J. Natl. Cancer Inst. **92**, 1472-1489.

50. Stattin, P., Bylund, A., Rinaldi, S., Biessy, C., Dechaud, H., Stenman, U.H., Egevad, L., Riboli, E., Hallmans, G., & Kaaks R. (2000) J. Natl. Cancer Inst. **92**, 1910-1917.

51. Kaplan, P.J., Mohan, S., Cohen, P., Foster, B.A., & Greenberg, N.M. (1999) Cancer Res. **59**, 2203-2209.

52. Satariano, W.A., Ragland, K.E., & Van Den Eeden, S.K. (1998) Cancer. **83**, 1180-1188.

53. Nicholson, D.W. (2000) Nature. **407**, 810-816.

54. Ahmad, N., Gupta, S., Husain, M.M., Heiskanen, K.M., & Mukhtar, H. (2000) Clin. Cancer Res. **6**, 1524-1528.

LEGEND FOR FIGURES

Figure 1: Effect of GTP infusion on prostate cancer development in TRAMP mice evaluated by longitudinal MRI analysis. **(A)** MRI was used to assess the growth of primary tumor in TRAMP mice followed longitudinally in individual animal. Details are described in 'Materials and Methods' section. A marked reduction in prostate development was observed in these mice after 0.1% (w/v) GTP infusion between 8 to 32 weeks. Representative images of **(a, b)** non-transgenic, **(c, d)** water-fed TRAMP mice and **(e, f)** GTP-infused TRAMP mice are shown here at 20 **(a, c, e)** and 30 **(b, d, f)** weeks of age. Arrows indicate prostate. **(B)** Volumetric analysis of the TRAMP mice prostate after GTP infusion. The data is represented as percent change in relative pixel density observed at 20 and 30 weeks of age where non-transgenic mice prostate is taken as control. Values represent mean $\pm$ SE of 5 animals. *p <0.001 compared to TRAMP water-fed.

Figure 2: Effect of GTP infusion on serum levels of **(A)** IGF-I and **(B)** IGFBP-3 in TRAMP mice. Eight-week old TRAMP mice were infused with 0.1% GTP (w/v) as sole source of drinking fluid for 24 weeks. Blood was withdrawn at 8, 16, and 24 weeks after GTP infusion and serum IGF-I and IGFBP-3 levels were analyzed by enzyme linked immunoabsorbant assay. Details are described in 'Materials and Methods' section. A marked inhibition in serum IGF-I and restoration in serum IGFBP-3 levels were observed after GTP infusion. Values represent mean $\pm$ SE of 10 animals. *p <0.001 compared to TRAMP water-fed.

Figure 3: Effect of GTP infusion on GU-apparatus and prostate histology in TRAMP mice. **(A)** Photograph of typical GU-apparatus of TRAMP mice exhibiting hyper-proliferation, **(B)** GU-apparatus of TRAMP mice with 0.1% GTP infusion (w/v) for 24 weeks. A marked decrease in GU-weight and volume was observed in TRAMP mice after GTP consumption. **(C)** Histologic

examination of a typical TRAMP mouse prostate at 32 weeks of age revealed moderately-differentiated neoplastic cells with extensive cribriform structures, marked thickening, remodeling and hypercellularity of the fibromuscular stroma. Magnification=40X **(D)** 0.1% GTP infusion (w/v) to TRAMP mice resulted in a marked reduction in epithelial stratification, cribriform structures, the glands remain simple, without epithelial thickening or surface complexity. Magnification=40X. Representative figures are shown here.

Figure 4: Effect of GTP infusion on the protein expressions of PCNA in the TRAMP mice prostate. **(A)** Protein expression of PCNA by Western blot, and **(B)** immunohistochemical analysis is shown here. In water-fed TRAMP, an extensive PCNA staining was observed in the nuclei. 0.1% GTP infusion (w/v) resulted in the marked reduction in the protein expression of PCNA in these mice. Equal loading of the protein in the lanes was confirmed by stripping the membrane and reprobing it with β-actin. Details are described in 'Materials and Methods' section.

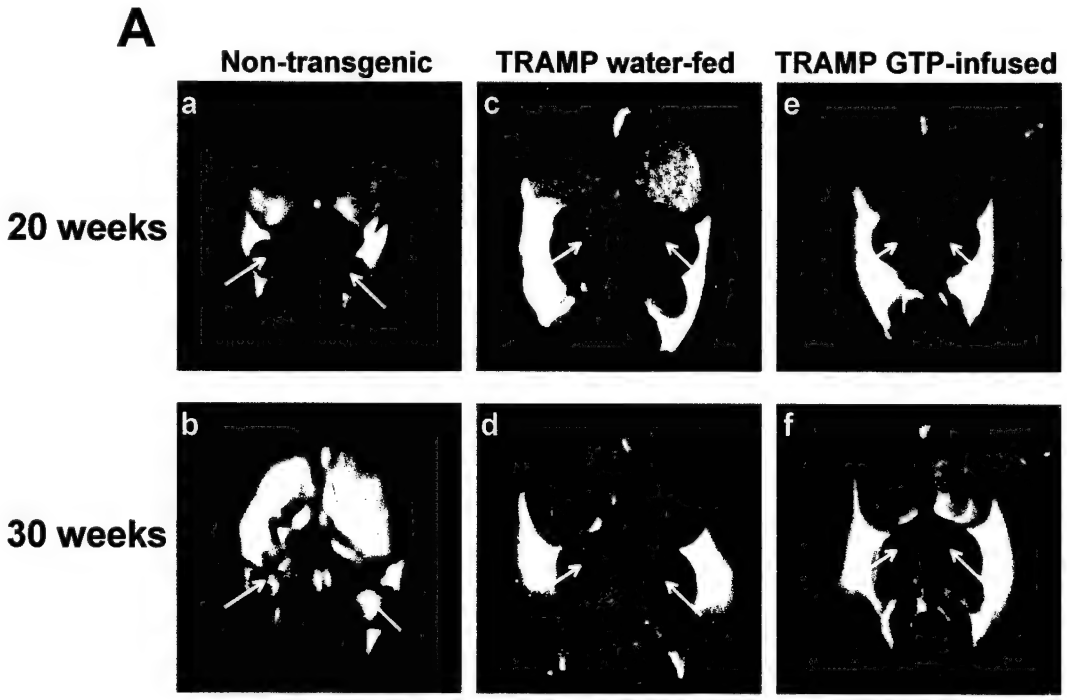
Figure 5: Effect of GTP infusion on the extent of apoptosis in the TRAMP mice prostate. **(A)** Apoptosis was determined by cell-death ELISA^{PLUS} as per vendor's protocol. Data are expressed as Enrichment factor. Values represent mean $\pm$ SE of 10 animals. *p <0.0001 compared to water-fed TRAMP mice. **(B)** Immunofluorescence detection of prostate tissue in water-fed and GTP-infused TRAMP mice by M30 CytoDEATH antibody that binds to caspase cleaved epitope of the cytokeratin 18 cytoskeletal protein, a marker of apoptosis. A marked increase in M30 fluorescence was observed after 0.1% GTP infusion (w/v), compared to water-fed TRAMP mice. A representative figure from each group at 80X magnification is shown here. Details are described in 'Materials and Methods' section.

Figure 6: Effect of GTP infusion on **(A)** Tumor free survival and **(B)** Survival probability in TRAMP mice. A significant increase in tumor free survival ($p<0.001$, log-rank test) and survival probability ($p<0.001$, log-rank test) in GTP-infused TRAMP was observed.

Table 1: Effect of oral infusion of GTP on the morphology of prostate and genito-urinary (GU) weight in TRAMP mice and their non-transgenic littermates[a]

Group[b]	Number of Animals	Palpable Tumor[c]	Animals with Metastasis[d]				Prostate weight (mg)	GU-weight (g)
			Lymph	Lungs	Liver	Bone		
Experiment 1								
Non-TG Control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	17.4 ± 1.4	0.45 ± 0.05
TRAMP water-fed	10	10/10	9/10 (90%)	7/10 (70%)	4/10 (40%)	3/10 (30%)	68.2 ± 8.4	3.76 ± 0.48
TRAMP GTP-infused	10	3/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	32.6 ± 2.8*	1.08 ± 0.12*
Experiment 2								
Non-TG Control	10	0/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15.6 ± 1.2	0.52 ± 0.04
TRAMP water-fed	10	10/10	10/10 (100%)	6/10 (60%)	4/10 (40%)	2/10 (20%)	83.7 ± 11.6	4.36 ± 0.58
TRAMP GTP-infused	10	4/10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	36.2 ± 3.8*	1.20 ± 0.14*
Cumulative								
Non-TG Control	20	0/20	0/10 (0%)	0/20 (0%)	0/10 (0%)	0/10 (0%)	16.5 ± 1.8	0.49 ± 0.05
TRAMP water-fed	20	20/20	19/20 (95%)	13/20 (65%)	8/20 (40%)	5/20 (25%)	76.0 ± 10.8	4.06 ± 0.64
TRAMP GTP-infused	20	7/20	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	27.5 ± 3.2*	1.14 ± 0.16*

[a] The data represented in each experiment is the mean ± SE of 10 mice.
[b] Mice (8 weeks of age) received plain drinking water (control group) or GTP (0.1% w/v) infusion in drinking water for 24 weeks. At the age of 32 weeks, the animals were sacrificed and studied for prostate tumorigenesis and metastases.
[c] Prostate tumor was assessed by abdominal pelvic palpation.
[d] Metastases in the lymph, liver and bone was examined under the microscope while metastasis in lungs was examined by the India ink method. Details are described in 'Materials and Methods'.
* $p < 0.001$, water-fed, control TRAMP compared with GTP-infused TRAMP, Student's 't' test.



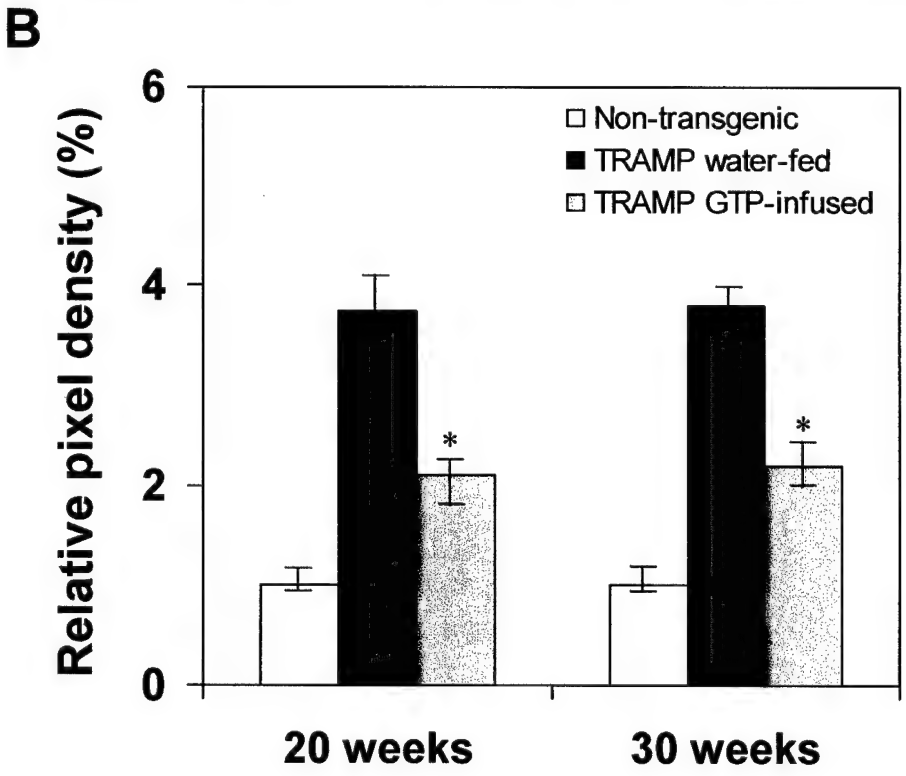


Figure 1 **Gupta *et al.***

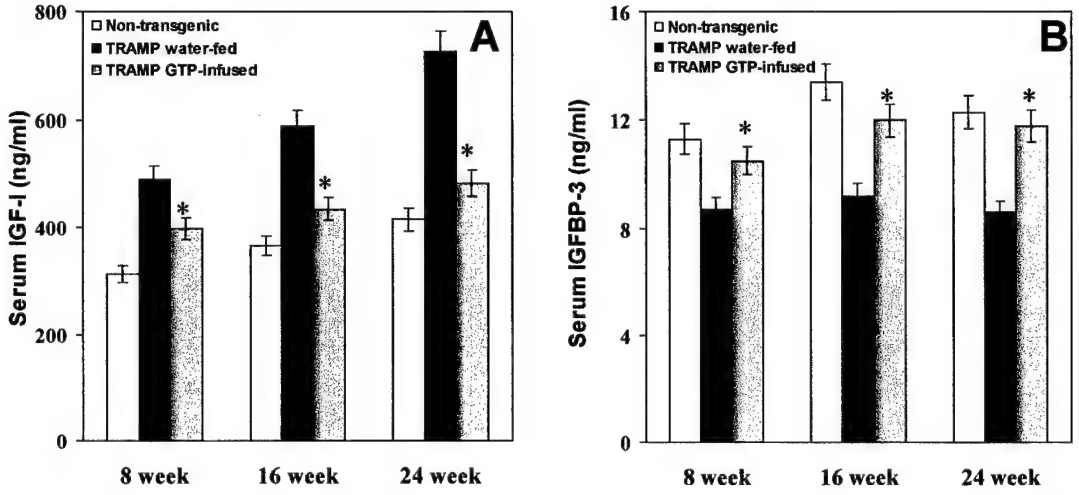


Figure 2 **Gupta *et al.***

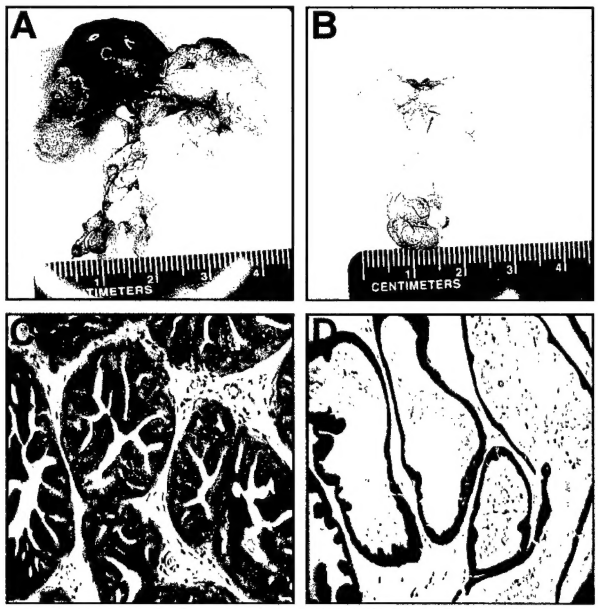
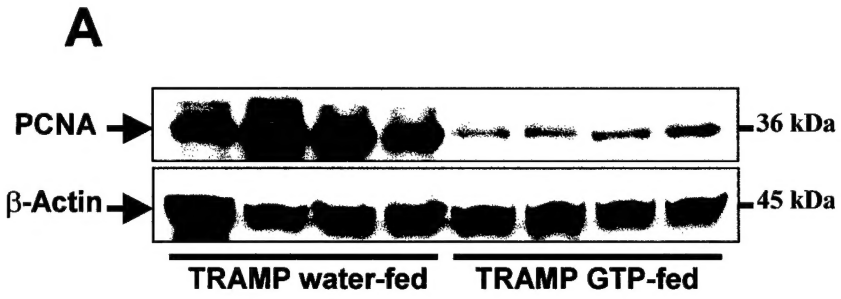


Figure 3 **Gupta *et al.***



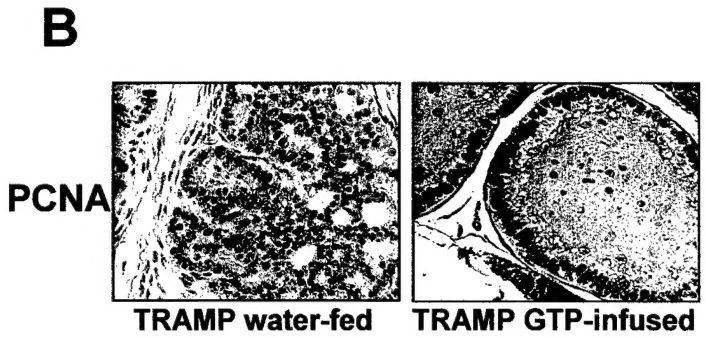


Figure 4 **Gupta *et al.***

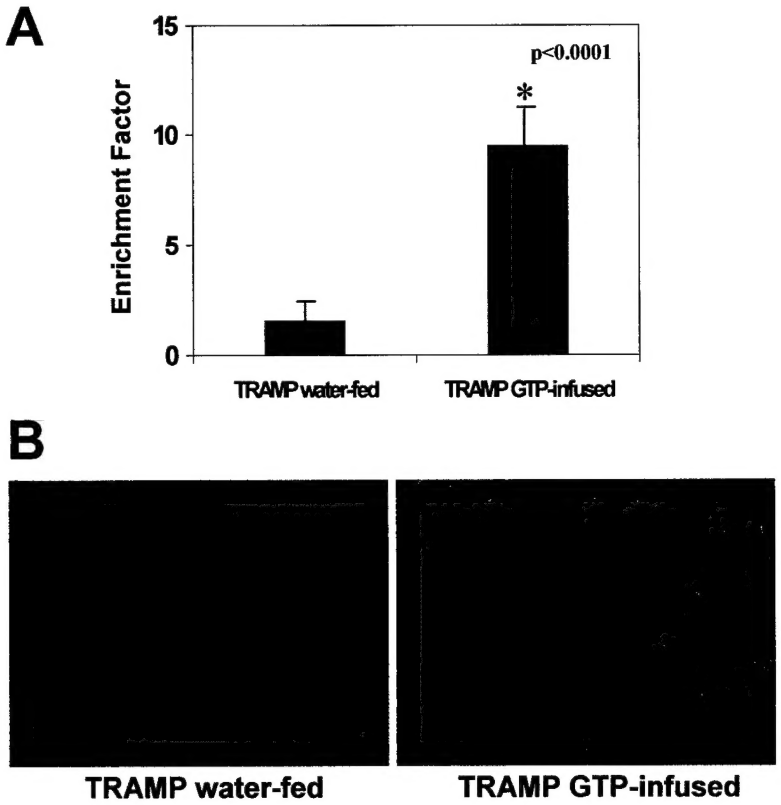
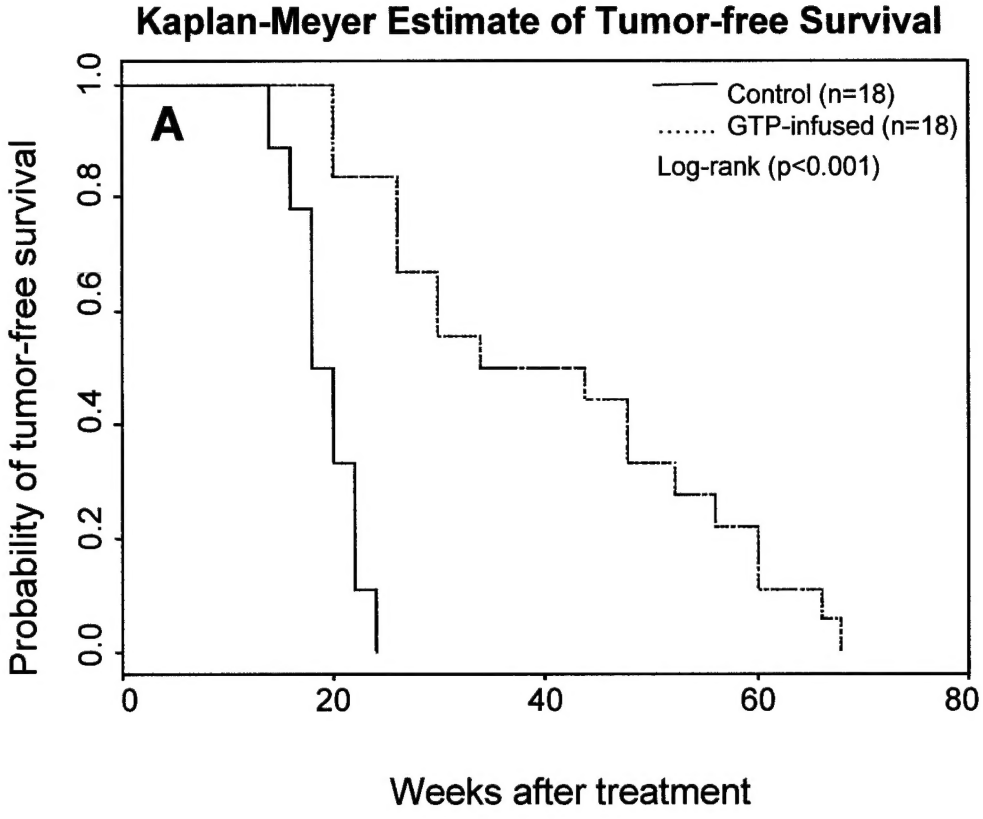


Figure 5 **Gupta *et al.***



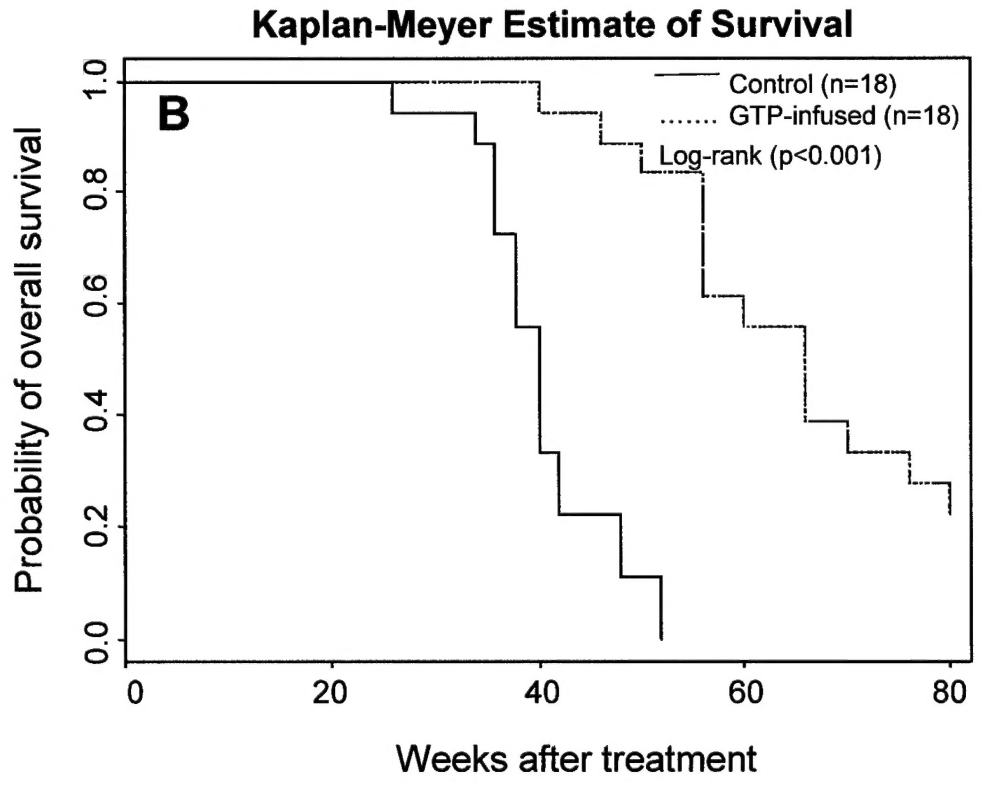


Figure 6 **Gupta *et al.***